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Evolution of Models of Homologous Recombination

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Abstract With the elucidation of the structure of DNA in 1953, it became possible to think in molecular terms about how recombination occurs and how it relates to the repair of DNA damage. Early molecular models, most notably the seminal model of Holliday in 1964, have been followed by a succession of other proposals to account for increasingly more detailed molecular biological information about the intermediates of recombination and for the results of more sophisticated genetic tests. Our current picture, far from definitive, includes several distinct mechanisms of DNA repair and recombination in both somatic and meiotic cells, based on the idea that most recombination is initiated by double-strand breaks.

Abbreviations

DSB	double-strand break
dHJ	double Holliday junction
BIR	break-induced replication
SDSA	synthesis-dependent strand annealing
PMS	post-meiotic segregation
Ab4 : 4	aberrant 4 : 4 segregation
SSA	single-strand annealing

1

Introduction

In humans and other vertebrates, the repair of DNA damage by homologous recombination is essential for life. In addition, recombination is essential for the proper segregation of chromosomes in meiosis and for the generation of genetic diversity. Moreover, defects in DNA repair by homologous recombination are strongly correlated with many types of human cancers. For all these reasons, as well as for the purely intellectual pleasure of understanding these processes, the development of molecular models to explain homologous recombination has been an exciting area of study. In this review I focus on mostly genetic results that have driven the construction of molecular models of recombination; however, these models have been increasingly influenced by our growing understanding of the biochemical properties of gene products required to carry out recombination. The reader seeking more details concerning the actions of recombination proteins is directed to many recent review articles (Aylon and Kupiec 2004; Cahill et al. 2006; Cox 2003; Haber

2006; Krogh and Symington 2004; Kuzminov 1999; Lusetti and Cox 2002; O'Driscoll and Jeggo 2006; Raji and Hartsuiker 2006; Sung and Klein 2006), including other chapters in this BOOK or the accompanying volume in this SERIES. This review is necessarily historical, but when recent insights help to understand certain concepts, time warps occur.

1.1

Prelude

Before there was an understanding that the chromosome consisted of DNA, there was a fascination with the mechanisms by which homologous chromosomes could undergo crossing-over. Early ideas emerged from studies in *Drosophila* and maize. Even before cytologically identifiable homologous chromosomes were used to establish definitively that genetic recombination was indeed accompanied by a reciprocal exchange of chromosome segments (Creighton and McClintock 1931; Stern 1931), there was speculation how recombination might take place. Janssens (1909) imagined that pairs of homologous chromosomes must break and join, but how such pairs of breaks could be made to ensure that the recombined chromosomes had not lost any genes was difficult to imagine. Belling (1933) instead suggested that the newly copied chromatids could have undergone exchange through some sort of copy-choice mechanism as new chromatids were generated.

In a remarkable essay, Muller (1922) focused on the “synaptic attraction” between homologous chromosomes, likening it to the assembly of a crystal—a prescient anticipation of base-pairing! How recombination might happen was suggested from Muller’s studies of X-irradiated chromosomes, which established the idea that chromosome breaks could be joined in novel ways to produce chromosome rearrangements (Muller and Altenburg 1930). Irradiation could also lead to apparently reciprocal exchanges between homologous chromosomes in mitosis and there was therefore the possibility that meiotic recombination might occur by some sort of breaking and joining. The finding that crossovers arising in meiosis were distributed non-randomly along the chromosome, exhibiting crossover interference, suggested that the mechanism of exchange was highly regulated (Muller 1916; Sturtevant 1915).

By the time the DNA structure was elucidated, it became evident that understanding the molecular nature of the gene and its functions, including recombination, would come—also as predicted by Muller (1922)—from the study of unicellular organisms, first in both bacteria and bacteriophage and then in fungi. In fact, before DNA was known to be a double helix of base-paired strands, Hershey and Chase (1951) had seen clear evidence of a hybrid bacteriophage chromosome in which one recombinant chromosome could yield both mutant and wild-type offspring for a particular gene. About 2% of the individual phage arising from this cross, when plated on a bacterial lawn, gave mottled plaques, which Hershey and Chase interpreted as evidence that

the genetic material was “heterozygous” at that locus. With the realization in 1953 that DNA was a double helix, it was possible to interpret these “heterozygotes” as evidence of hybrid DNA, with one strand carrying one allele and the complementary strand carrying the other (Levinthal 1954).

The study of meiosis in fungi was stimulated by the advantages of being able to recover all four haploid products of meiosis, as each spore would germinate into a colony; thus all four DNA strands of two recombining homologous chromosomes would be recovered (Fig. 1). The first important insight that opened the way to investigate the mechanism of recombination was made

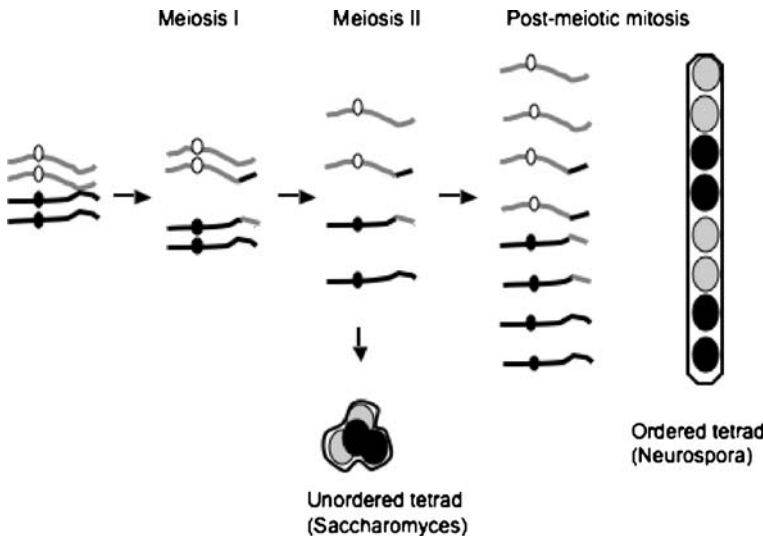


Fig. 1 Analysis of products of meiosis in ascospores. Following recombination at the 4-chromatid stage of meiosis, the four chromatids segregate, similar to what occurs in mammalian male meiosis. In budding yeast and other organisms with unordered tetrads the four nuclei are packaged into four spores within an ascus. Selective digestion of the ascus cell wall allows the micromanipulation of spores on an agar plate so that all four spores germinate. The resulting colonies can be scored for nutritional requirements, drug-resistance, growth at high temperature, and other attributes by replica plating them to different media or conditions. In *Neurospora* and other filamentous ascomycetes, there is a post-meiotic mitotic division, producing eight nuclei that are packaged into spores. In some organisms these asci are ordered, such that the position of the centromeres of each pair of homologous chromosomes are reflected in the linear order of the spores. Spore shape and spore color can be scored directly without microdissection and subsequent replica plating. A heterozygous marker (Aa) that has not undergone any crossing-over relative to its centromere will be seen as a first-division segregation (AAAAaaaa) pattern, whereas a meiosis in which there has been a single exchange between the marker and the centromere will have a second-division segregation pattern (AAaaAAaa). Gene conversions and post-meiotic segregations can be seen directly for visible markers in eight-spored ordered tetrads or after replica plating spore colonies to see sectorized colonies

by Lindegren (1953), who found evidence of nonmendelian segregation of markers. Instead of always obtaining 2 wild-type: 2 mutant segregation for a carbon utilization gene, he found some tetrads with 3 : 1 or 1 : 3 patterns. To describe this phenomenon, Lindegren invoked the term *gene conversion*, first coined by Winkler in 1931 (Lindegren 1958). Gene conversions appeared to be *non-reciprocal* transfers of genetic information, very different from the reciprocal exchange events in crossing-over.

The primitive state of the *S. cerevisiae* genetic map precluded Lindegren from showing what had happened to nearby markers, but Mitchell (1955) studying *Neurospora* was able to show that while one marker was displaying nonmendelian segregation, flanking genetic markers segregated 2 : 2. Thus gene conversion was a local recombination event rather than a problem affecting an entire chromosome arm. Mitchell also noted that gene conversions and crossing-over in a small interval were correlated, and Freese (1957) went further to suggest that they were the consequence of a single event. An elegant proof that gene conversions were bona fide non-reciprocal transfers of the original alleles (rather than new mutations) was provided by Fogel and Mortimer (1969).

It took several more years before two other types of nonmendelian segregation pattern—post-meiotic segregation (PMS)—were appreciated. These were first seen in fungi in which meiosis was followed by a post-meiotic mitotic division prior to spore formation, leading to the ordered arrangement of 8 spores reflecting the orientation of the centromeres at the time of the first meiotic division. An ascus with no crossover or gene conversion between the marker and its centromere would give a “first division segregation” pattern (++) ++ -- --); a crossover between the marker and its centromere yielded second division segregation (++) ++ -- ++ --). A 6 : 2 gene conversion appeared as (++) ++ ++ --). Olive (1959) found the segregation of a gray-spore (*g*) allele of *Sordaria* included not only 4 : 4 and both 6 : 2 and 2 : 6 asci (i.e., those expected for gene conversion) but also asci with 5 : 3 and 3 : 5 segregation, in which one meiotic product had given rise to one mitotic copy with the *g* allele and the other with *G* (i.e., ++ ++ +- --). These outcomes were reminiscent of the “heterozygous” results in bacteriophage crosses. Subsequently Kitani et al. (1962) found the last important nonmendelian segregation pattern of so-called aberrant 4 : 4 (Ab4 : 4) asci (++) +- -+ --).

Kitani et al. (1962) also made another fundamentally important observation. Among asci that exhibited 6 : 2, 2 : 6, 5 : 3, 3 : 5 or Ab4 : 4 segregation, about 36% had also undergone a reciprocal crossing-over between adjacent markers that flanked the aberrantly segregating *g* locus. In contrast, among all tetrads the two markers showed only 4% crossing-over. Moreover, in almost all of the cases, a chromatid that exhibited PMS was one of the two chromatids involved in the crossover event. These observations suggested that crossing-over and these nonmendelian segregation events were intimately connected, and that the process of crossing-over often generated heterodu-

plex DNA. A similar conclusion was reached by Fogel and Hurst (1967); in budding yeast, with four spores, the appearance of 5 : 3 and 3 : 5 types could be seen by careful replica plating of the original spore colonies such that one half of the colony would be wild-type and the other half auxotrophic for some nutritional marker. Consequently, budding yeast data are also discussed in terms of 8 DNA strands.

1.2

The First Molecular Models of Recombination

Several early models imagined that gene conversions arose by template switching during the pre-meiotic replication of homologous chromosomes (Freese 1957; Lissouba et al. 1962; Stadler and Towe 1963). Although these “switch” or “copy-error” models could account for gene conversion and crossing-over, they did not offer explanations of PMS outcomes. One influential model, based on density analysis of recombinant bacteriophage, was the “copy-choice” mechanism proposed by Matthew Meselson and Jean Weigle (1961). Their model suggested that the end of a broken molecule could be unwound and that each strand of a broken chromosome end could base-pair with complementary sequences of an intact DNA duplex. Strand pairing then promotes copying of the template, producing a nonreciprocal crossover product (Fig. 2). This model contained apparently the first representation of the 4-strand branched intermediate now called a Holliday junction (HJ). We will return to ideas about break-copy recombination near the end of the review, when we examine mechanisms of recombination-dependent DNA replication, also known as break-induced replication.

Break-copy ideas were almost immediately confronted with data supporting break-join recombination. In the same year that Meselson and Weigle proposed break-copy, Kellenberger et al. (1961) used density-gradient analysis of phage λ parents of different densities, combined with ^{32}P labeling of one parent to show that most recombination involved a physical exchange of DNA with little new synthesis (Anraku and Tomizawa 1965).

In 1962, Robin Holliday (1962) briefly speculated that recombination might involve junctions of parental DNA molecules that contained heteroduplex DNA. Moreover, extrapolating from recent findings of template-directed repair of UV-induced lesions, Holliday conjured up the idea that mismatches in heteroduplex DNA could be repaired in a somewhat analogous fashion. Such repair, he noted, could account for gene conversions.

Soon after, Harold Whitehouse (Whitehouse 1963) provided the first illustrated molecular models that would use heteroduplex DNA to create a reciprocal exchange between two DNA molecules. Whitehouse suggested two variations of his model (Fig. 3). In both cases he suggested that single-strand DNA breaks could occur in adjacent DNA molecules, either at different points (Fig. 3A) or at the same point (Fig. 3B), but in strands of opposite polar-

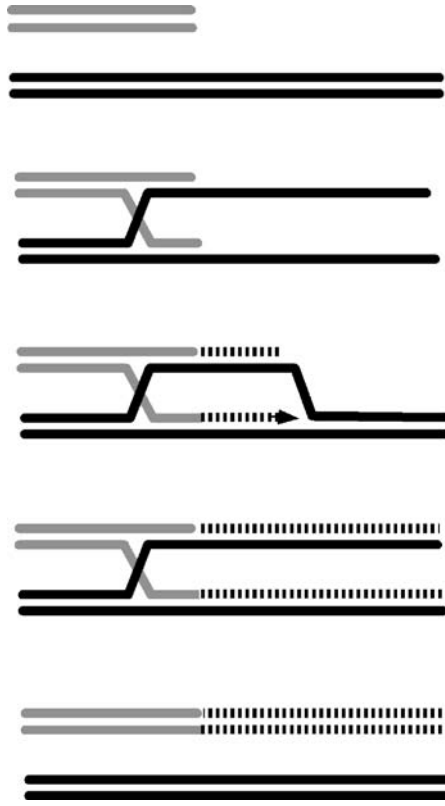


Fig. 2 Meselson and Weigle's 1961 Break-Copy recombination mechanism. The two strands of a broken chromosome fragment can form base pairs with an intact template and promote copying to the end of the template, thus producing a recombined, full-length product

ity. In the first model, the nicked single strands could unwind and pair together to form hybrid (heteroduplex) DNA. Subsequently the gaps created by the formation of the heteroduplex could be filled in by new DNA synthesis. Whitehouse then suggested that there would be "another cycle of strand separation and hybridization, degradation of surplus DNA, and finally correction of mismatched base pairs." In the second model (Fig. 3B), each of the initially displaced strands would pair with a newly copied version of the opposite homolog, again creating regions of heteroduplex DNA at the crossover point. The last step involved the removal of part of two "old" strands of DNA to complete the crossover structure. The heteroduplex regions could then be subject to some type of repair of mismatches to account for various nonmendelian ratios of alleles among the meiotic products. During the completion of the recombination event, there were additional patches of new synthesis; these could yield gene conversion events without being directly associated with a crossover.

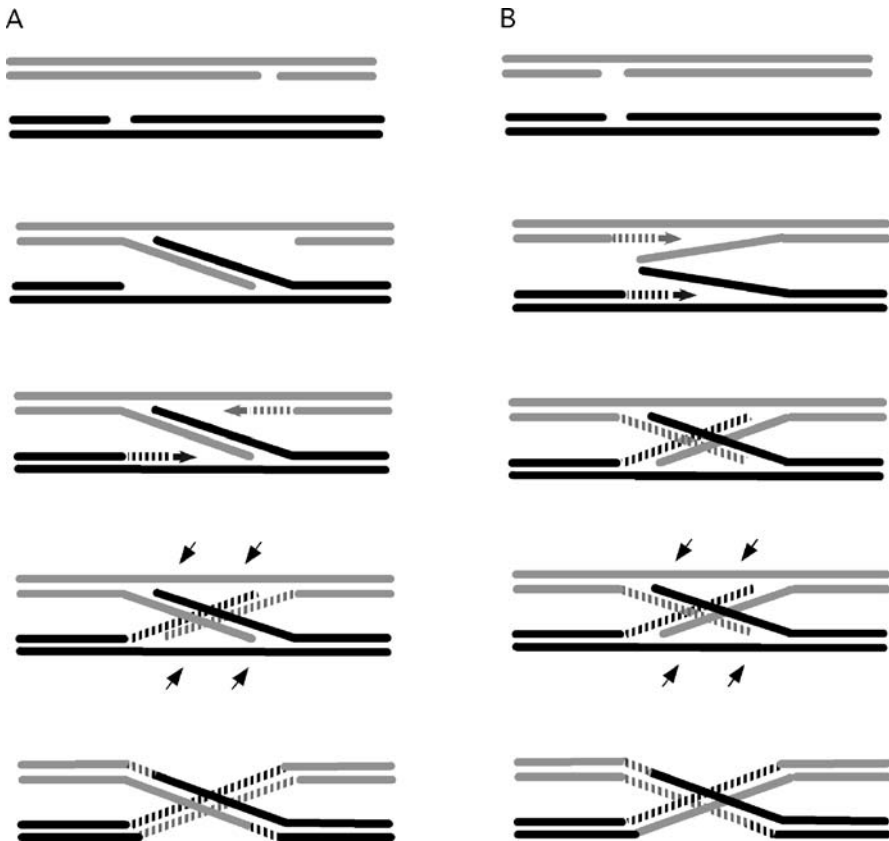


Fig. 3 Whitehouse's 1963 models. **A** Nicks at different locations in strands of opposite polarity allowed annealing and joining of two DNA molecules by a region of heteroduplex DNA. New DNA synthesis, strand displacement and annealing creates a second cross-connection, again with heteroduplex DNA. The "extra" strand of DNA is excised and degraded (indicated by *arrows*), leaving a crossover. Completion of DNA synthesis to join all strands results in flanking regions in which there are 3 strands of one parental type, allowing gene conversions to be made without an immediate crossover. **B** A similar process involving strands of the same polarity and where the nicks occur at the same position. Here heteroduplex is formed between old and newly synthesized strands

2

Robin Holliday's Remarkable Model

Robin Holliday's 1964 model (Holliday 1964) created a much simpler and elegant molecular view of recombination that accounted for all of the key findings made by his predecessors. Holliday envisioned that crossing-over began with a coordinated pair of single-strand nicks—but on strands of the same polarity—on a pair of homologous chromosomes. These nicked strands

could be unwound and displaced, allowing an exchange of single strands, accounting for the formation of regions of heteroduplex DNA that might cover a region where the DNA differed between the homologous chromosomes (Fig. 4). This reciprocal exchange of single DNA strands led to the creation of

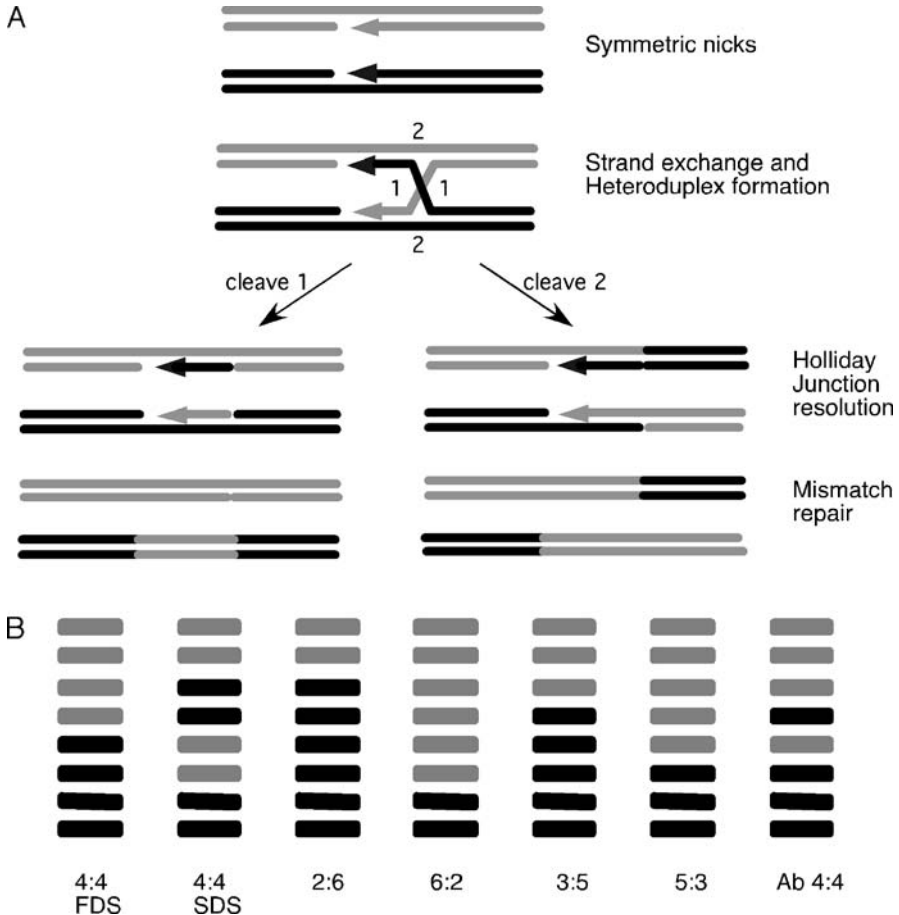


Fig. 4 Holliday's 1964 model. **A** A pair of nonsister chromatids after meiotic DNA replication are shown; the two other chromatids, uninvolved in recombination, are not shown. A pair of same-strand nicks leads to a reciprocal exchange and formation of symmetric heteroduplex connected by a 4-stranded symmetric structure now known as a Holliday junction (HJ). The HJ can be cleaved by cutting either of two pairs of strands (orientations 1 and 2). Crossovers occur when the HJ is cleaved so that only the crossing-strands connect the two homologous chromosomes. In the example shown, mismatch corrections lead to a 6 : 2 gene conversion. **B** Heteroduplex regions can be converted, restored or left unchanged depending on the efficiency of mismatch correction. All types of non-mendelian segregation patterns can be accounted for by this mechanism, as shown here for an ordered tetrad

the four-stranded structure—what we now call a Holliday junction—which could be resolved to give both crossover and noncrossover outcomes. The second key idea, drawn from his 1962 speculations, was that mismatch repair of heteroduplex DNA could produce aberrant ratios of alleles among the progeny, including both gene conversions and post-meiotic segregations (Fig. 4B).

Combining the idea that Holliday junctions could be resolved either with or without crossing-over with the idea that heteroduplex intermediates could be restored, converted or left unrepaired, Holliday set out a mechanism that accounted for all of the results obtained in various fungal systems. Over time, however, as more data accumulated, it became clear that—in detail – the proportions of various outcomes expected from Holliday’s model did not fit the observed types of tetrads recovered from several different fungi. Consequently, Holliday’s model has undergone several important evolutionary modifications that will be discussed below, but the three ideas that he emphasized—the creation of heteroduplex DNA by the exchange of a single strand of DNA, the formation of a branched intermediate Holliday junction and the mismatch correction of heteroduplex DNA—remain the foundation of our present understanding.

2.1

Strand Exchange by Single-Strand Annealing

Soon after Holliday’s model appeared, Charles Thomas (1966) offered a slightly different view in which all of the outcomes would be linked to reciprocal crossing-over (Fig. 5A). In Thomas’ model, staggered nicks would occur on both strands of each duplex molecule and the separation of strands would permit the formation of reciprocally recombined molecules, linked by regions of heteroduplex DNA. This mechanism of *single-strand annealing* (SSA) could work even if all the nicks were not at precisely the same position, because gaps or overhanging single-stranded segments could be enzymatically filled in or clipped off, respectively. We will return to a discussion of SSA towards the end of the review, but in the case where SSA occurs following a double-strand break.

2.2

Evidence Favoring Holliday’s Model: Hotspots and Gradients of Gene Conversion

Evidence supporting several features of Holliday’s model came from more intensive analysis of gene conversion events within individual genes. In the ascomycete *Ascobolus immersus* Jean-Luc Rossignol and his colleagues had isolated many alleles within genes affecting spore color (Rossignol 1969). Some alleles showed a high rate of nonmendelian segregation, with as many as 5% of the asci containing a gene conversion; other alleles had conversion

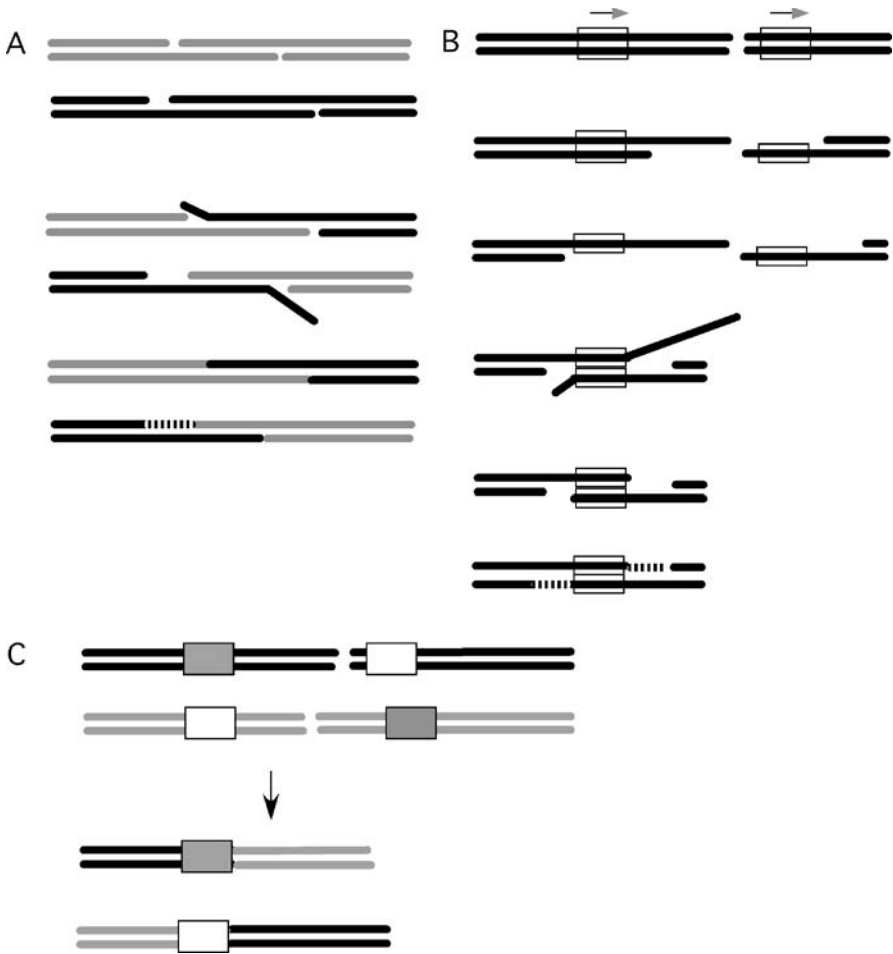


Fig. 5 Single-strand annealing. **A** Charles Thomas' SSA model to obtain reciprocal recombination by annealing overlapping single-strands of DNA from two chromosomes with offset nicks on both strands. **B** DSB-induced SSA leading to an intrachromosomal deletion between directly oriented, non-tandem repeats. The DSB ends are resected by 5' to 3' exonucleases and Rad52-mediated annealing between flanking homologous sequences can occur, even in the absence of Rad51. Long 3' ended ssDNA tails can be cleaved off and the missing DNA filled in by using the 3' ends of the paired strands as primers. **C** Reciprocal crossovers (translocations) created by SSA can be accomplished if there are a pair of DSBs flanking pairs of homologous sequences

rates 10 times lower. When the rate of nonmendelian segregations of each allele, crossed to wild-type, was plotted versus the position of each allele within the gene, it became apparent that there was a distinct gradient, with most alleles showing high levels of nonmendelian segregation at one end (Lissouba et al. 1962; Rossignol 1969).

As more alleles were obtained it became clear that some high-frequency gene conversion alleles yielded primarily 6 : 2 or 2 : 6 patterns whereas other alleles gave 5 : 3 and 3 : 5 patterns, with some 6 : 2 and 2 : 6 (Leblon 1972a,b). Similarly there were both types among infrequently converting alleles. A similar conclusion was reached for alleles of the *arg4* locus in *S. cerevisiae* (Fogel et al. 1979; Mortimer and Fogel 1974)¹.

The gradient of gene conversion along a gene could be explained if there were a *hotspot*—a preferential site of initiation of the recombination—at one end of the gene. This could be the site of DNA strand cleavage. The probability that heteroduplex DNA formation resulting from strand exchange would include an allele within the gene would be roughly proportional to the distance between the hotspot and the allele. Thus the probability that nonmendelian segregation would occur would also be proportional to the distance of the allele from the site of initiation of recombination.

2.3

Challenges to the Holliday Model

The Holliday model provided a conceptual basis for understanding the kinds of tetrads that arose in various fungi and was completely consistent with what little was known about recombination in higher organisms, but further analysis of fungal genetic data began to present examples where the observed patterns of segregation were inconsistent with the outcomes expected from Holliday's model. There were two major concerns. First, whereas Holliday's model imagined *symmetric heteroduplex* DNA (that is, where both chromatids involved in the recombination event form equivalent heteroduplex DNA), the data reviewed below were more consistent with a recombination intermediate that had only one heteroduplex region (that is, *asymmetric heteroduplex*). Second, Holliday's model suggested that all the crossover events should be located at the end of the heteroduplex DNA opposite from the point where the strands were nicked and unwound. This, too, proved not always to be the case.

2.4

The 5 : 3 Paradox

In Holliday's strand exchange model, the most frequent types of non-mendelian segregations are 6 : 2 and 2 : 6 gene conversions that would be expected if one heteroduplex region was converted and the other was restored to its initial genotype. This suggests that in general conversion and restoration are equally likely to occur. Now consider 5 : 3 tetrads in *Neurospora* or

¹ During this period that recombination models were being developed, their authors took into account recent experimental findings that had been presented and discussed at meetings long before they made their way into print—in contrast to current practice where data are often only presented at meetings if they are in press or published.

Ascobolus. Whereas budding yeast tetrads are not ordered, the octads in *Neurospora* or *Ascobolus* are ordered such that the pair of sister centromeres and the subsequent mitotic copies of each chromatid are always adjacent. Hence ordered tetrads can display two distinct 5 : 3 patterns: ++ ++ +- -- and ++ +- ++ --. According to Holliday's model, both of these outcomes should arise by mismatch correction of the same ++ +- +- -- intermediate. In the ++ ++ +- -- case, we imagine that one heteroduplex is restored and the second is left unrepaired. With ++ +- ++ --, we imagine that one heteroduplex is unrepaired and the second is converted. The Holliday model would predict that both types of outcomes would be equally probable because each arises from correction of one of the two regions of heteroduplex; but data in *Ascobolus* from Stadler and Towe (1971) as well as additional data from others showed that this was not the case. In one experiment, the ++ ++ +- -- pattern was found 53 times compared to a single example of ++ +- ++ --. We can imagine two explanations for this asymmetry. First, it could be explained if restoration is rare and conversion is frequent. Alternatively, the data can easily be understood if usually there is only a single heteroduplex DNA region formed during recombination.

2.5

An Absence of Double-Crossovers

By the same token, if gene conversion were efficient, then one would expect a high frequency of what appear to be double crossover events. For example, consider the case where the Holliday junction is resolved without a crossing-over, and each heteroduplex in a ++ +- +- -- intermediate is converted to the genotype of the invading strand, to produce a ++ -- ++ -- ascus. Relative to the flanking markers, this outcome appears as if a double crossover has taken place, but such outcomes proved to be extremely rare in all species of fungi that were examined. If one argues from the example above that restorations are rare and conversions are frequent, there should be many of the apparent double crossovers. However, one would not expect to find many such double crossovers if most of the time there was asymmetric heteroduplex DNA.

2.6

Alleles that Show a High PMS Fail to Show a High Proportion of Aberrant 4 : 4 Asci

Holliday didn't specify any particular mechanism by which heteroduplex DNA would be restored or converted or whether different alleles would have intrinsically different properties of being converted, restored or left unrepaired. This question was answered by Rossignol and his colleagues, who collected an impressive number of different alleles of the *b2* locus in *Ascobolus*, using mutagens that caused either single base pair substitutions or small, most likely 1-bp, in-

sertions and deletions (Leblon 1972a). (One mutagen they used was similar to that used to carry out the famous frame-shift experiment in phage T4 that showed that the genetic code in bacteria was composed of 3-bp codons.)

Mutations in *b2* cause changes in spore color, determined by the haploid spore genotype, so hundreds of asci can be scored visually. When crossed to a wild-type strain, some alleles yielded many 6 wild-type: 2 mutant and few 2: 6 segregants; others yielded few 6: 2 and many 2: 6 outcomes. Other alleles yielded many 5: 3 and 3: 5 asci (Paquette and Rossignol 1978). By genetic mapping, Paquette and Rossignol were able to show that each type of allele was not clustered in one part of the *b2* gene; one could have high PMS alleles (5: 3 and 3: 5) that mapped very close to both high 6: 2 and high 2: 6 alleles (Rossignol et al. 1979). Rossignol postulated that the different types of alleles represented different types of mutations. In heteroduplex between wild-type and a frameshift allele that resulted from the insertion of a single base pair (termed + 1), the mismatch could be preferentially corrected in favor of the insertion. In contrast, heteroduplex DNA involving a 1-bp deletion would be preferentially repaired in favor of the (1 bp larger) wild-type DNA (Rossignol and Paquette 1979). In the type of intermediate postulated by Holliday, a cross between wild-type and a + 1 frameshift (designated **a**) would be preferentially corrected from ++ +**a** +**a** **aa** to ++ **aa** **aa** **aa**, thus producing many 2: 6 and few 6: 2 asci.

Conversely, an intermediate with a - 1 frameshift (designated **b**) would be corrected from the ++ +**b** +**b** **bb** intermediate to ++ ++ ++ **bb**, that is, to yield mostly 6: 2 asci.

Curiously, however, with many of the poorly corrected (type **c**) alleles, although there were many 5: 3 and 3: 5 tetrads (without any bias), there were few aberrant 4: 4 cases—far fewer than one would expect if one simply multiplied the probabilities of two sites each being left unrepaired. This absence of Ab4: 4 is not the expected outcome if there are two heteroduplex regions arising by reciprocal strand exchange and if the type **c** allele is frequently not repaired. Of course it was possible that there was some special kind of repair system operating, that would always repair one heteroduplex and leave one unrepaired, but perhaps the assumption that there were two heteroduplex regions was not generally correct.

Taken together, the data outlined above all argued that most gene conversion events were best described by creating an intermediate of recombination with only one heteroduplex DNA region.

3

Molecular Models Based on a Single Initiating DNA Lesion

In the early 1970s two geneticists offered ways to imagine how recombination could be initiated not by a pair of lesions—one on each chromatid—but by

a single initiating event. Paszewski (1970) imagined that a nicked strand in one DNA molecule could invade an intact duplex and initiate new DNA synthesis from its 3' end (Fig. 6A). A subsequent, sequential pair of nicks would

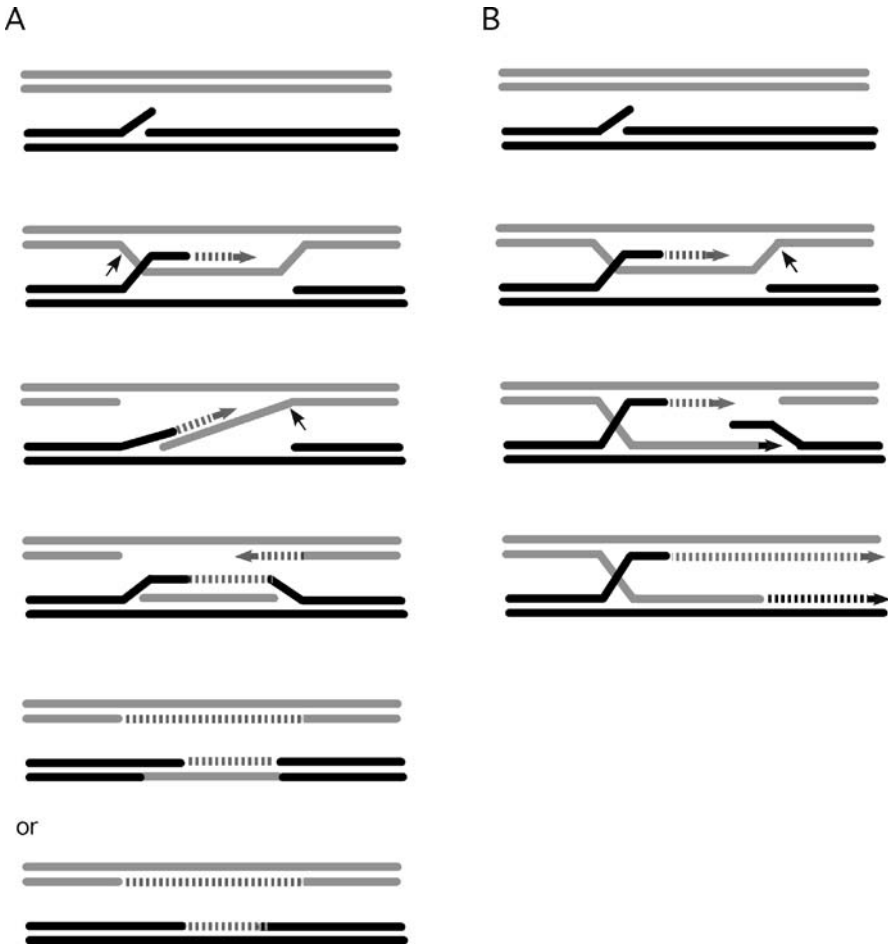


Fig. 6 Two early models of recombination induced by a single-strand nick. **A** Paszewski's 1970 model. A single nick provokes strand unwinding and strand invasion, prompting new DNA synthesis. The displacement loop (D-loop) is cleaved (*small arrow*), leaving a connection between the homologs by a heteroduplex DNA region. A second nick and a rejoining step creates a novel triplex structure at the recipient locus that can be resolved either to leave heteroduplex DNA or a gene conversion. The template chromosome is restored by fill-in DNA synthesis. **B** Hotchkiss' 1974 mechanism. A nick leads to strand invasion while the nick is enlarged by exonucleases into a gap. The D-loop is cleaved, allowing the displaced strand to anneal with the ssDNA in the gapped region. Further 5' to 3' exonuclease removes the remaining broken strands and new DNA synthesis proceeds to the end of the template

lead to a duplex segment of DNA that could reassociate with the original DNA molecule while the resulting gap could be filled in, leaving the donor molecule unchanged. The novel triplex structure at the recipient could be resolved in two ways, one leading to a gene conversion and the other leaving a single region of heteroduplex DNA. How this type of event might also lead to crossovers was not indicated.

Rollin Hotchkiss (1971, 1974) suggested a simpler model also starting with a single nicked DNA (Fig. 6B). Again, a strand invasion would initiate new DNA synthesis, but here the displaced strand could anneal with initially nicked molecule, where the nick was enlarged into a gap by an exonuclease. The two 3' ends could be extended to the end of the DNA molecule. Here there are actually two heteroduplex regions, but one of them is short and the other longer. How the branched structure would be resolved was not addressed.

4

The Meselson–Radding Model (1975)

Stimulated by a meeting on recombination in Aviemore, Scotland, where much of the information mentioned above was reviewed and discussed among the participants, Matthew Meselson and Charles Radding proposed a new model of recombination (Meselson and Radding 1975), sometimes called the Aviemore model. Meselson and Radding proposed that only one chromatid was nicked, to initiate recombination (Fig. 7). The 3' end of the nick could be used as a DNA primer, in much the same way as repair synthesis occurs after removal of UV-induced cyclobutane dimers. In this case, the movement of the recombination-promoting DNA polymerase displaced a single-strand of DNA, analogous to the initiation of rolling-circle DNA synthesis during bacterial conjugation. The displaced 5'-ended strand then somehow located and invaded the homologous sequence of another chromatid, by breaking the base pairs of the intact DNA and allowing base pairing between one strand and the invading complementary strand. Strand invasion created a displacement or "D" loop, as suggested earlier (see Fig. 6).

Most likely strand invasion required the activity of a recombination protein. (Note that the bacterial RecA protein, known genetically to be a key factor in recombination, was not purified until several years later (McEntee et al. 1979; Ogawa et al. 1979; Shibata et al. 1979).) After strand invasion, an unknown nuclease was invoked to cut the displacement loop (D loop), resulting in a single region of heteroduplex DNA and two molecules held together by what might be called a half-Holliday junction. This would account for the asymmetric nature of heteroduplex DNA in most meiotic tetrads, but it did not explain how crossing-over would occur.

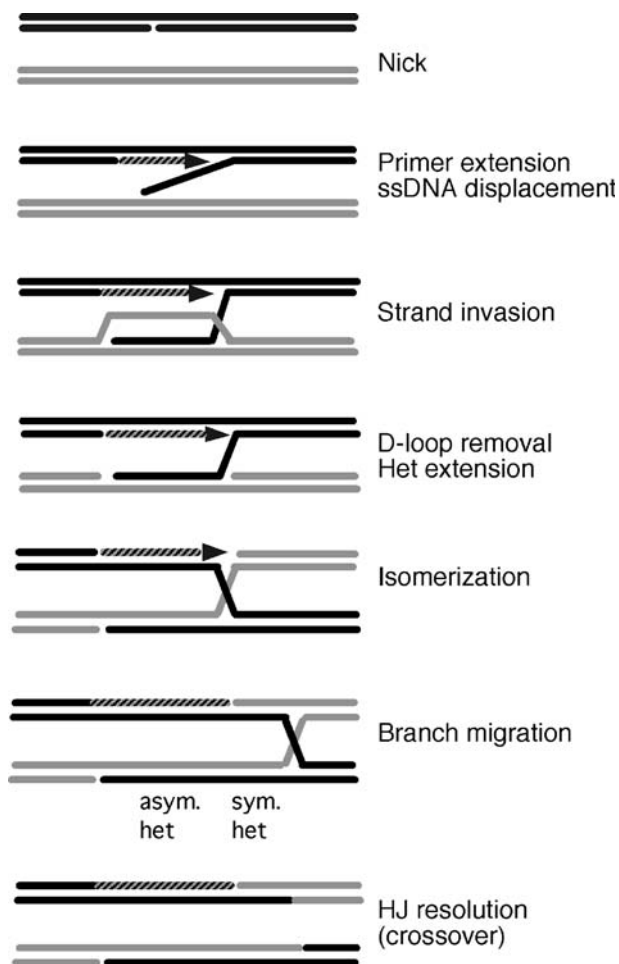


Fig. 7 Meselson and Radding's 1975 model. A single nicked strand is displaced by new DNA synthesis primed from the 3' end of the nick. The displaced strand can form a region of heteroduplex DNA by strand invasion and the formation of a displacement loop (D-loop). Cleavage of the D-loop leaves a single region of heteroduplex DNA adjacent to an HJ that is always distal from the initiating nick. Isomerization of the HJ and subsequent branch migration leads to the formation of a symmetric region (sym. het) of heteroduplex adjacent to an asymmetric (asym. het) segment (with only one heteroduplex region), still with the crossover point far from the initiating lesion

If the non-crossed strands were cleaved by a nuclease, one molecule would be recombined for flanking genetic markers and there would be one intact strand and a region of heteroduplex DNA to hold the joint molecule together, but the expected reciprocal crossover molecule would be in two pieces, and one would have to invent a special ligase that would put the ends together, without loss of any DNA sequence. Meselson and Radding had an alternative

proposal. They suggested that the half-crossover intermediate could *isomerize* into a symmetrical Holliday junction (Fig. 7). In this way, a complete Holliday junction replaces the half-crossover, which could then be resolved either as a crossover or as a noncrossover in the manner that Holliday had envisioned.

One way one might envision the isomerization process is that the left-hand side of the structure remains fixed but the two DNA molecules on the right side have been picked up and flipped over, but, in fact, if you do this with a physical model of the Holliday junction, you find that the crossed strands get twisted. This problem was addressed by Sigal and Alberts (1972), who saw that isomerization had to occur in two steps, first by creating an “open” intermediate structure by a half rotation of the HJ (which produces a completely symmetric structure in which all base pairs can be formed) (Fig. 8B) and then by rotating a different set of arms in a half-rotation.

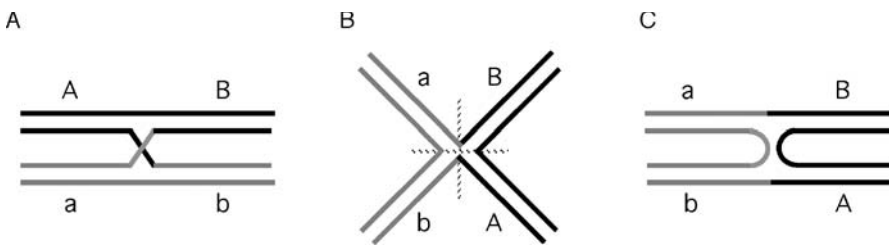


Fig. 8 Holliday junction configurations. **A** Conventional view of a HJ resulting from the Holliday model. **B** An “open” HJ that emphasizes its inherent symmetry, so that cleavage of two of the four strands will result in either crossovers or noncrossovers. **C** The most stable HJ structures in vitro, unconstrained by proteins, may have homologous sequences in a *trans* configuration. In three dimensions, the stacked double-helices do not lie exactly parallel in the plane of the drawing, but form a right-handed, antiparallel X-structure (McKinney et al. 2003, 2005). In this configuration, branch migration can ensue from pulling either A and a or B and b away from the junction

Biophysical studies of synthetic HJ have suggested that the most stable structures—in the absence of proteins—are not those that would seem most applicable to crossovers between chromosomes; rather than having homologous chromosome arms (A and a) in *cis*, A and a are found in *trans* (Fig. 8C) (Duckett et al. 1988; McKinney et al. 2003). It is likely that this structure, though more stable in solution, is changed in the presence of the proteins that bind to, stabilize, and cleave HJs in vivo (Bennett and West 1995).

4.1

A Transition from 5 : 3 to Ab4 : 4 Tetrads:

Branch Migration of a Holliday Junction can Produce Symmetric Heteroduplex

The Meselson and Radding model also took advantage of a special feature of the Holliday junction: it can migrate along two double-stranded DNA

molecules without expending any net energy. Hydrogen bonds between two base pairs must be broken and the two DNA molecules rotate by one base pair and then reform two new base pairs, with different partners. If this process continues, the branch can move down the DNA, leaving in its wake two heteroduplex DNA regions (Fig. 7). *Branch migration* provided a new way to create heteroduplex DNA. Without some driving force, branch migration is as likely to remove such regions as it is to extend them; but as we will see later, we now know there are proteins that can facilitate branch migration and potentially give it direction (Shinagawa and Iwasaki 1996; West 1997).

Thus recombination could begin with a single heteroduplex region. An isomerization would produce a complete Holliday junction. Then branch migration would create a region of *symmetric* heteroduplex. In this way, the frequent aberrant 4 : 4 tetrads obtained in *Sordaria* and *Ascobolus* could be accommodated. As discussed below, branch migration may also be important for the process of resolving Holliday junctions as well.

4.2

Evidence Supporting the Meselson–Radding Model: One or Two Heteroduplex Regions Within a Gene

A further investigation of the *b2* locus by Rossignol and his colleagues revealed still other curious features of meiotic recombination. As mentioned above, most poorly repaired type *c* alleles gave many 5 : 3 and 3 : 5 asci but very few Ab4 : 4 cases, but there was a subset of type *c* alleles that did in fact produce a significant number of Ab4 : 4 asci, along with 5 : 3 and 3 : 5 types. Genetic mapping of these alleles within the *b2* gene revealed that all of these mutations were found at one end of the gene, apparently furthest from the hotspot (Rossignol et al. 1979). Rossignol postulated that there was some sort of transitional event during recombination so that the molecular intermediates switched from an asymmetric heteroduplex (which could only produce 5 : 3 or 3 : 5 asci) to *symmetric* heteroduplex, where Ab4 : 4 types could appear (Rossignol et al. 1984). The isomerization step of the Meselson–Radding model (Fig. 7) seemed to provide a molecular basis for this transition.

4.3

More Evidence: a Large Heterology Apparently Blocks Branch Migration

Rossignol then provided a very compelling demonstration that one could block this transition if one of the two homologous chromosomes had a large insertion or deletion in the middle of the gene (Hamza et al. 1981; Langin et al. 1988a,b). If one looked at a poorly repaired marker at the end of the gene where Ab4 : 4 tetrads were found, the presence of the large discontinuity in one parent nearly abolished Ab4 : 4 tetrads but did not diminish 5 : 3 or 3 : 5 events. A simple way to explain this was that a single heteroduplex could

form even across a large heterology, but branch migration would be blocked by a large insertion/deletion.

5 Problems with the Meselson–Radding Model

5.1

Where are the Crossovers?

Fogel et al. (1979) provided another critique of both the Holliday and Meselson–Radding models. They realized that a poorly repaired allele could be used as a marker to learn about the position of the crossover. They assumed that there was a specific point of initiation of gene conversion at one end of the gene; this was seen by the fact that there was a clear gradient in the level of gene conversions for alleles distributed along the gene. In 1979,

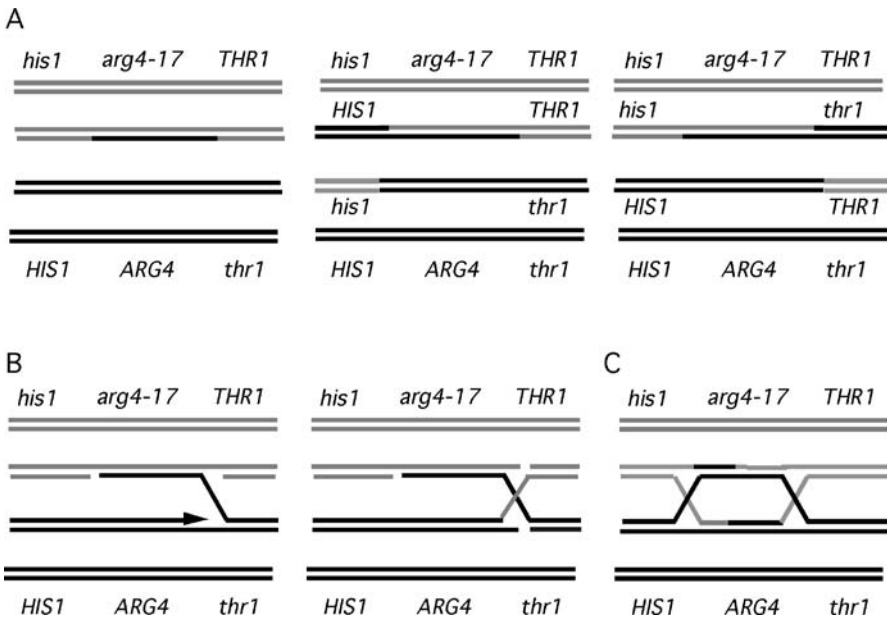


Fig. 9 Locating the position of crossing-over relative to an unrepaired heteroduplex. **A** Tetrads 3 : 5 for *arg4-17* could be parental for flanking markers (left) or could be tetratypes for flanking markers with a crossover in the *his1* to *arg4-17* interval (center) or in the *arg4-17* to *THR1* interval (right). The Meselson–Radding model (**B**) would predict that all crossovers would be one side, depending on location of the initiating nick, but in fact crossovers are found of both sides. **C** Crossovers on either side of a region of heteroduplex can be easily accommodated by the double Holliday Junction DSB repair model of Szostak et al. (1983). Parts of the Fig. were modified from Mortimer and Fogel (1974)

before cloning and DNA sequencing of yeast genes, it wasn't known that the high end of the gradient was quite often at the 5' end of the gene. Most alleles, such as *arg4-16* or *arg-19*, gave mostly 6 : 2 and 2 : 6 gene conversions, but Fogel focused on the *arg4-17* allele, which yielded many 5 : 3 and 3 : 5 tetrads. Some of these nonmendelian segregations were tetratype with respect to the flanking markers *his1* and *thr1*. Contrary to the expectation of the Holliday or Meselson–Radding models, that the crossovers should be located at the end furthest from the site(s) of initiation, two types of tetrads with 5 : 3 segregation of *arg4-17* and tetratype for flanking markers were obtained, with roughly equal frequencies (Fogel et al. 1979; Mortimer and Fogel 1974) (Fig. 9).

5.2

Hotspots Appear to be Eliminated by Gene Converted

Another concern about the Meselson–Radding model came from studies of fission yeast, *Schizosaccharomyces pombe*, where Gutz (1971) had identified a “hot” allele, *ade6-M26*, that when crossed with wild-type, gave many more nonmendelian meiotic events than were seen with other alleles closely linked to M26 (see G Cromie and GR Smith, this BOOK). A curious feature of this allele was that it “self-destructed”—that is, most gene conversions yielded 6 : 2 outcomes in which the “hot” allele was lost. Of course this one exceptional example could be explained in several ways (preferential mismatch correction of a heterology, for example), but one explanation was that the recombination process created a lesion at the hot spot that required replacement of the DNA that initiated the recombination event. Such an outcome would not be predicted by the Meselson–Radding model, where the hotspot should be faithfully recopied and DNA at or adjacent to this site would be displaced to invade and create heteroduplex with a wild-type sequence.

6

Alternative Ways to Initiate Recombination

6.1

Several Provocative Suggestions

During the 1970s there were several other provocative and inventive suggestions as to how joint molecules containing single or double Holliday junctions might form, even in the absence of an initiating nick or DSB. Among these was the branch migration model by Broker and Lehman (1971) that a pair of nicks on strands of opposite polarity could lead to strand unwinding and the pairing of the two nicked ends, leaving the two un-nicked strands to pair as well (Fig. 10A). The intermediate is a Holliday junction. Subsequent branch

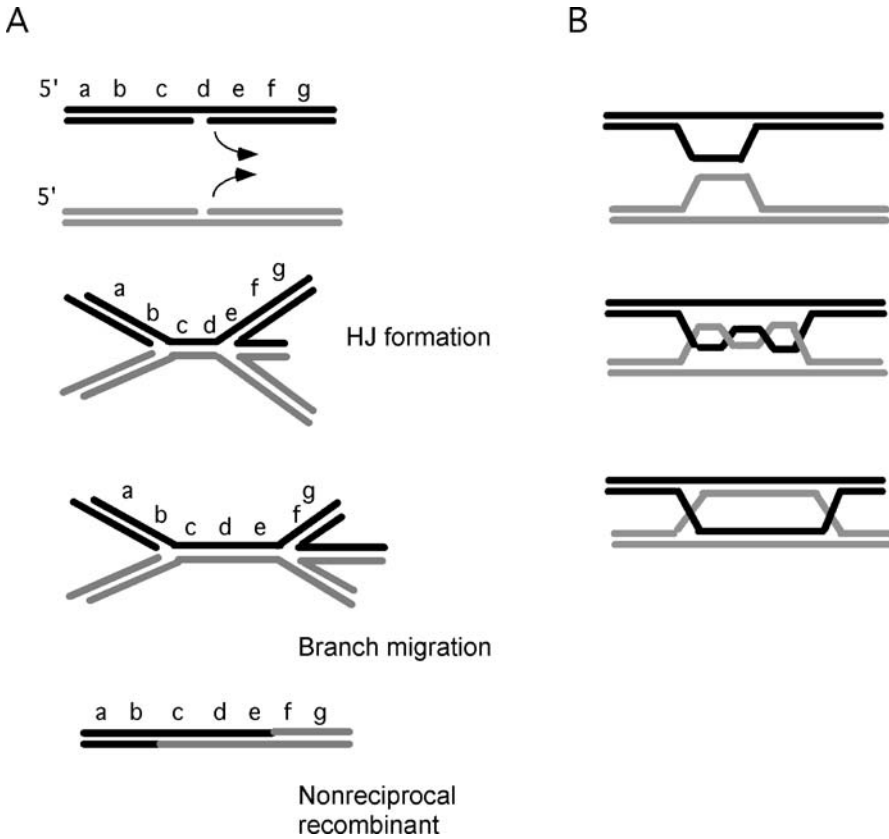


Fig. 10 **A** The branch-migration model of Broker and Lehman (1971) begins with the nicking and denaturation of DNA strands such that alternative pairing produces a Holliday junction. Subsequent branch migration creates a long region of heteroduplex DNA. The branched molecule is resolved into various recombinant structures by single-strand cleavages and/or by exonucleolytic digestion of some strands. **B** Strand annealing of a denatured region on two chromosomes, aided by topoisomerase-driven interwindings can produce a double Holliday junction (Champoux 1977). An intermediate showing interwindings is shown, but at the end both pairs of complementary strands are in duplex, B-form DNA

migration of the HJ could enlarge the paired structure and, with additional nicks or exonucleolytic digestion, could yield a variety of nonreciprocal recombinants. In recent years an analogous formation of a HJ by dissociation and pairing of strands has been invoked to account for stalled and regressed replication forks, creating a “chicken foot” (a HJ) (Lopes et al. 2003; Michel 2000).

Henry Sobell (1972) suggested that palindromic regions of DNA could form single-stranded hairpin structures, allowing the two homologous chromosomes to become paired through these regions after a pair of nicks in

complementary loops promoted base-pairing. In a series of steps extensive formation of heteroduplex, followed by ligation of the original nicks would lead to a dHJ (the reader is invited to peruse the original paper to follow the choreography). Without ligation, intermediates analogous to those proposed by Broker and Lehman would be generated.

James Champoux (Champoux 1977), based on his studies of topoisomerases, suggested a simple model for initiating recombination in which local denaturation of two helices, aided by topoisomerases, could intertwine two duplexes in the absence of any DNA break (discounting the transient openings and closings demanded by topoisomerases) to form a covalently closed dHJ (Fig. 10B). This idea was further elaborated by Dressler and Potter (1982) in their important review article.

John Wilson (1979) also proposed a “nick-free formation of reciprocal heteroduplex.” His suggestion involved rotation of bases in the minor groove to form quartets of base pairing, producing a pair of tightly associated, intercoiled heteroduplexes. The resulting structure is a length of “fused heteroduplex” with 4 double-stranded arms. If this structure were nicked, an open dHJ with “separated heteroduplexes” would result. The ends of the fused region are intrinsically sites of crossing-over.

One other provocative idea from this period was Frank Stahl’s suggestion that a donor region could become over-replicated to provide extra copies of DNA that could be used to effect gene conversion by a pair of crossover events without the formation of much heteroduplex DNA (Stahl 1979). Stahl’s book contained a number of other ideas that stimulated much discussion and culminated in his collaboration with Szostak, Orr-Weaver and Rothstein in a comprehensive model based on double-strand breaks (Szostak et al. 1983).

6.2

The First Recombination Model Based on Double-Strand Breaks

Michael Resnick (1976) was concerned with explaining the repair of DSBs induced by ionizing radiation. Genetic studies suggested that damage produced by ionizing radiation could stimulate heteroallelic recombination and that some recombination events were crossover-associated. Based on the known polarity of phage λ exonuclease, Resnick presciently proposed that the ends of a DSB could be processed by a 5′ to 3′ exonuclease, producing 3′-ended tails (Fig. 11A). One processed end could then base-pair with a complementary strand of an intact duplex, which was, he imagined, cleaved so that it could pair easily with the resected DSB end. Then the 3′ end of the invading strand could be used as a primer to initiate a short region of new DNA synthesis. If this extended strand was displaced from the duplex template and simply annealed to the opposite end of the DSB, the ends could now be re-sealed (and repaired). This simple mechanism is analogous to what is now referred to as

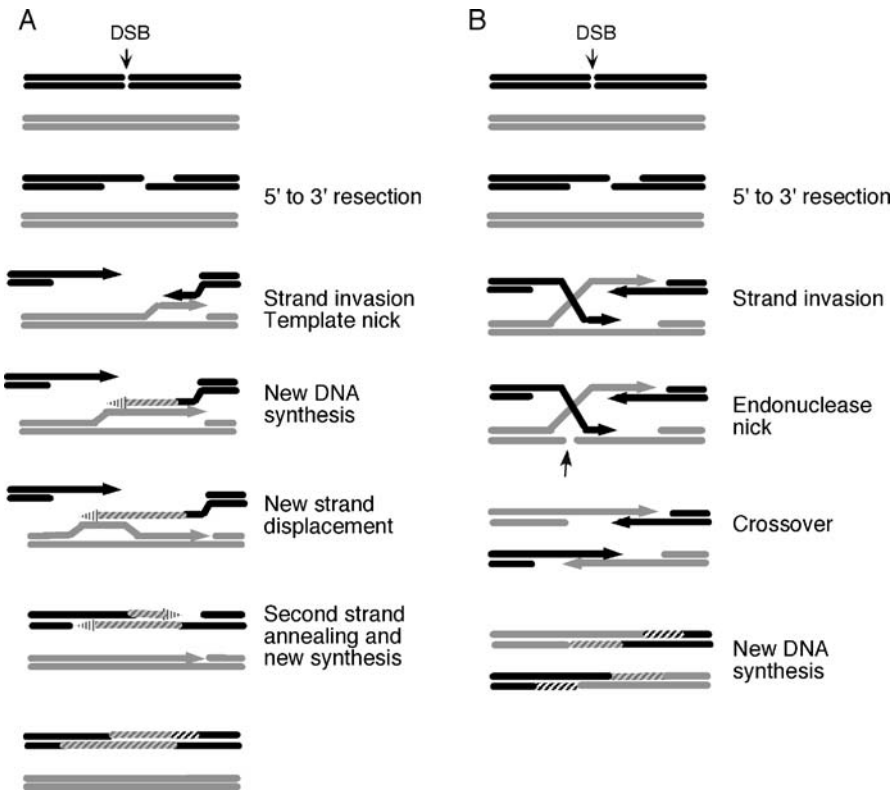


Fig. 11 The DSB repair model of Resnick (1976). **A** A DSB is resected and one of the 3' ends invades a template. The template strand is nicked. The paired DSB end initiates new DNA synthesis, which is displaced, allowing the original sequences to reanneal with the template. When the newly copied strand is long enough, it can anneal with the second end, priming a second round of new synthesis and the repair of the DSB. This leads to a noncrossover repair of the DSB. **B** Crossovers can be generated by DSB repair. Here, the nicked template strand itself can anneal with the single-stranded sequences created by 5' to 3' resection. Strand invasion of the original DSB end creates a Holliday junction. An endonuclease nick at the base of the HJ results in connections between the molecules that result in a crossover. DNA synthesis fills in the ssDNA gaps and leads to a crossover

synthesis-dependent strand annealing (SDSA), discussed further below. The Resnick model also involved the creation of a nick on the template strand, apparently to facilitate heteroduplex formation. This suggestion appears to have been made in the absence of knowledge of the D-loop that was invoked by the Meselson–Radding model, which appeared after the time Resnick had submitted his manuscript.

Resnick also provided a mechanism to account for repair events associated with crossing-over (Fig. 11B). Here, the nicked template strand is indeed

displaced and pairs with the opposite end of the resected DSB, creating a HJ containing a nick. Resnick postulated that this structure would be cleaved opposite the first nick by a second nick, resulting in a crossover. This idea precedes by several decades experimental evidence that the Mus81-Eme1 “resolvase” enzyme preferentially cleaves a nicked HJ in this fashion, discussed below (Gaillard et al. 2003; Osman et al. 2003).

Resnick’s model was published in the *Journal of Theoretical Biology* and was apparently either not seen or not appreciated by others working on mechanisms of recombination. Because his model dealt most specifically with ionizing radiation-induced DSBs and did not attempt comprehensively to relate the molecular mechanism to the body of data concerning meiotic recombination, Resnick’s model did not become part of the common parlance, despite its insights.

Resnick’s model, like Meselson–Radding’s, emerged before the first important findings about the enzymology of DNA repair and recombination were uncovered. It is beyond the scope of this review to delve deeply into the history of the discovery of the RecA protein; but it became much easier to think about the molecular mechanisms of recombination when there were purified proteins that could carry out strand exchange in vitro (McEntee et al. 1979; Ogawa et al. 1979; Shibata et al. 1979) (see C. Prévost, this BOOK). Researchers versed in genetics and biochemistry, and then molecular biology, began to devise new ways to test how recombination occurred.

6.3

A Key Experimental Transition:

Studying Recombination in Mitotic Rather than Meiotic Cells

Although the focus of recombination theorists had been on explaining meiotic recombination, as well as recombination in bacteria and bacteriophage, a decisive step in understanding the molecular basis of recombination came from studying transformation in budding yeast, by Terry Orr-Weaver, Jack Szostak and Rodney Rothstein (1981). Transformation of circular plasmid DNA carrying a selectable yeast gene such as *HIS3* is quite inefficient, although most transformants proved to have integrated the plasmid by an apparent crossing-over between the resident *his3* allele and the *HIS3* sequences on the plasmid. However, gene targeting was made much more efficient if the plasmid were cut with a restriction enzyme that cleaved somewhere in the *HIS3* sequence. In a plasmid carrying both *SUP3* and *HIS3* that could integrate either at *SUP3* or *his3*, cleavage in one homologous sequence resulted in essentially all the integrants at that location. Thus, double-strand breaks were very efficient in promoting homologous recombination.

A second key experiment carried out by the combined forces of the Szostak and Rothstein labs involved cutting out a segment of the homologous sequence so that each end of the cut plasmid could still recombine with *HIS3*

but the two ends were several hundred bp apart (Orr-Weaver and Szostak 1983; Orr-Weaver et al. 1981). The resulting integrations had two complete copies of the *HIS3* sequence. Thus there must have been “gap repair” (Fig. 12). These were clearly mitotic gene conversions in which the recombination event leading to the crossover-mediated integration of the transformed plasmid must have involved DNA synthesis so that the gap was replaced by a second copy of the template region.

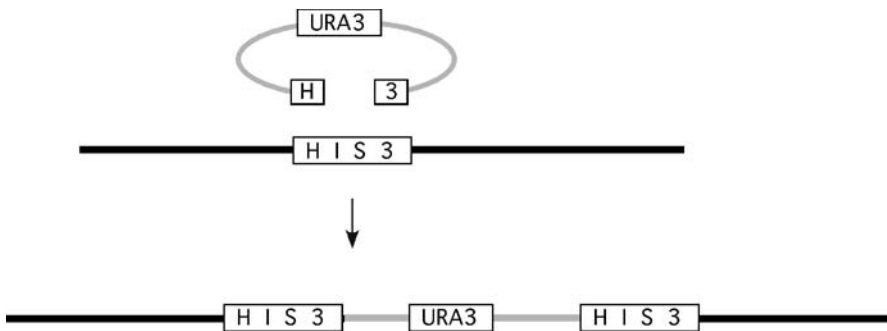


Fig. 12 Double-strand gap repair during plasmid transformation. A yeast plasmid, cleaved by restriction endonucleases to lack the middle portion of a gene, can integrate by recombination with the remaining homologous sequences. The resulting transformant has two intact copies of the targeted gene, thus indicating that the integration process involved new DNA synthesis to fill in the gap

Subsequently experiments were carried out with plasmids that could replicate autonomously. In this case repair could either occur with or without crossing-over, and both types of outcomes were found. It seems that the majority of events are not crossover-associated (Plessis and Dujon 1993) but there are many gene conversions accompanied by crossing-over; however, with a plasmid carrying a copy of ribosomal DNA, about half of the transformants were crossover-associated (Orr-Weaver et al. 1981).

7

The Double Holliday DSB Repair Model of Szostak, Orr-Weaver, Rothstein and Stahl

The Szostak et al. (1983) model (Fig. 13A) provided an explanation for the formation of mostly asymmetric heteroduplex on both sides of the DSB, but unlike the Meselson–Radding model, the double Holliday junction (dHJ) model also neatly accounted for the observation that a crossover accompanying nonmendelian segregation could occur on either side of the initiating lesion (see Fig. 9).

7.1

Processing of Double-Strand Break Ends

Like Resnick's 1976 model, the Szostak et al. model assumed that the DSB would be processed by 5' to 3' exonucleases to leave 3'-ended ssDNA regions. Several lines of evidence suggested that recombination involved ssDNA most likely with 3' ends. One influential experiment was carried out by White and Fox, using bacteriophage λ , that analyzed the types of heteroduplex DNA formed during recombination and deduced that heteroduplex DNA had 3' ends (White and Fox 1975). Moreover, the 3' end was appropriate to act as the primer of new DNA synthesis that would be needed for gap repair.

The original version of the Szostak et al. model was strongly influenced by their previous studies of DSB-mediated transformation in *Saccharomyces*, most especially by integrative transformation in which the linearized "ends-in" sequences were separated by a gap. The nonreciprocal transfer of DNA sequences during gap repair appeared to be the mitotic equivalent of a gene conversion event in meiosis. Hence in this double-strand break repair (DSBR) model, the DSB ends that initiated recombination were separated by a large gap and were resected to have relatively short regions of ssDNA that would perform strand invasion. This depiction arose from the assumption that most 6:2 and 2:6 gene conversions arose from gap repair rather than mismatch correction. 5:3 and 3:5 segregation was proposed to arise from asymmetric heteroduplex DNA that was not mismatch corrected. These assumptions were striking in view of the previous studies in *Saccharomyces* by Mortimer and Fogel and in *Ascobolus* from Rossignol's group from which it seemed clear that alleles that preferentially gave rise to 5:3 and 3:5 outcomes were interspersed with those yielding 6:2 and 2:6 gene conversions. Szostak et al. explained these observations by the assumption that the high-PMS alleles blocked enlargement of the gap and therefore were more often in heteroduplex DNA than in gaps. They made a similar argument, based on changing the activity of the resection of DSB ends, to explain *pms* post-meiotic segregation mutants that Williamson and Fogel (1985) had suggested were defective in mismatch repair.

The alternative explanation of Fogel's results was that there were at best small gaps and long regions of heteroduplex DNA and that differences in correction of different mismatches could readily explain the different types of nonmendelian segregation that appeared. White, Lusnak and Fogel (1985) later demonstrated that three *arg4* alleles that gave rise to 5:3 or 3:5 asci were indeed those that could form C:C mismatches which have proven in both prokaryotes and eukaryotes to be refractory to mismatch correction by the MutS/MutL mismatch machinery. Later, physical analysis of DNA extracted from meiotic cells showed that persistence of C:C mismatches whereas the reciprocal G:G mismatches were very rapidly repaired (Lichten et al. 1990). Moreover, the *pms* mutants of Williamson and Fogel indeed

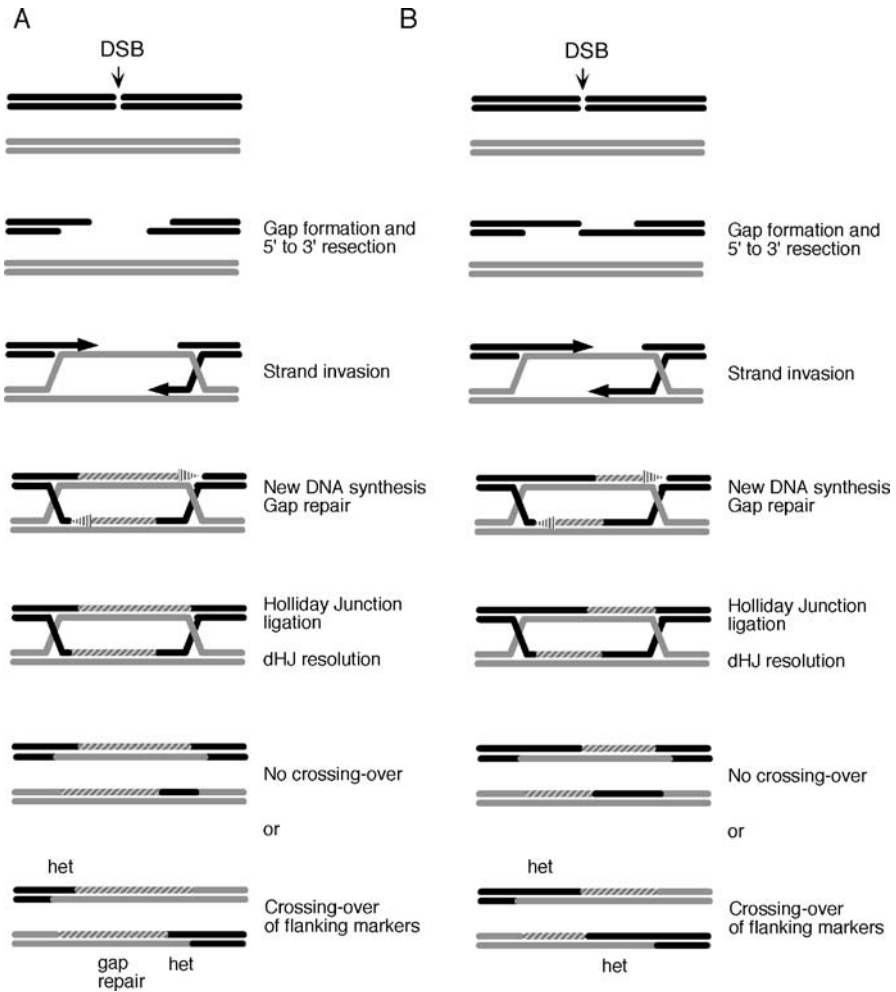


Fig. 13 The double Holliday junction (dHJ) model of Szostak, Orr-Weaver, Rothstein and Stahl (1983). **A** As originally proposed, a DSB was enlarged into a double-stranded gapped region, which was subsequently resected to have 3-ended single-strand tails that could engage in strand invasion. The first strand invasion would produce a D-loop to which the end of the second resected end could anneal. The initial structure has one complete and one half HJ, but branch migration of the half HJ allows the formation of a second complete HJ. New DNA synthesis completes the formation of a fully ligated structure that can be resolved into crossovers if the two HJs are cleaved in different orientations. Resolution of both HJs as crossovers should leave an apparent double-crossover in the middle; such outcomes are rare. Repair of the gapped region will inevitably lead to 6 : 2 or 2 : 6 events, whereas the short heteroduplex regions, if left without mismatch correction, would lead to 5 : 3 or 3 : 5 events. Coordinate branch migration of both HJs will produce only very short regions of symmetric heteroduplex that would be detected as Ab4 : 4. **B** The current view of the dHJ model has little or no gap-widening and much longer regions of heteroduplex (Stahl 1996)

proved to be mutations in the mismatch repair genes *PMS1*, *MLH1*, *MSH2*, *MSH6* and *MSH3* (Kolodner 1996; Kramer et al. 1989).

Further evidence against the presence of large gaps at DSB ends came from analysis of the DSBs themselves, showing that, at least on average, the ends were no more than a few nucleotides apart (Sun et al. 1991).

Over time, the DSB model has evolved to have little or no gap and much longer regions of heteroduplex DNA (Fig. 13B). It remains an open question whether some of the 3' ended ssDNA is resected or cleaved, so that at least small gaps could be an important feature of the mechanism. Very little is yet understood about the coordination of the two DSB ends in recombination.

7.2

The Double Holliday Junction

The second innovation of the DSB repair model was the assumption that there would be a fully ligated and symmetric double Holliday junction, rather than one full HJ and one single-strand half-crossover that would be the initial product of strand invasion and annealing of the second end to a D-loop. However, a small amount of branch migration of the "half-HJ" would make it possible to obtain two complete HJ. Filling-in and ligation would make a completely closed structure. Theoretically, each HJ could be cleaved independently and could yield either crossovers or noncrossovers. Crossovers would occur if one HJ were cleaved in a crossover mode and the second one were cleaved in a noncrossover orientation. If both HJ were cleaved in a noncrossover configuration, then there could be gene conversion without exchange. If both HJ were cleaved in the crossover mode, then a local double crossover should be seen (but it was already clear that these were rare). There has been very little analysis of the constraints imposed by a dHJ and how a resolvase would cleave such a structure, but we know that the bacterial RuvC Holliday junction resolvase, acting on purified yeast dHJs, yields roughly equal proportions of crossovers and noncrossovers from the dHJ structure (Schwacha and Kleckner 1995). Yet, as we will see below, the presence of meiotic recombination proteins in budding yeast appear to force resolution of dHJ almost always to give crossovers.

8

Identification of DNA Intermediates of Recombination

8.1

Physical Monitoring of Meiotic and Mitotic Recombination

Analysis of the kinetics of meiotic recombination was first achieved by Borts et al. (1984, 1986). They studied yeast meiotic recombination by using ho-

homologous chromosomes in which there were restriction endonuclease site polymorphisms flanking the region where crossing-over occurred. Consequently crossovers produced novel-sized restriction fragments. As expected from classical genetic experiments, the kinetics of appearance of the recombinant restriction fragments occurred after DNA replication was completed. This analysis also revealed that some meiosis-defective mutants such as *spo11* and *rad50* failed to produce any recombinants whereas others that did not yield viable spores (e.g. *rad6*) nevertheless permitted crossovers to appear.

About the same time, Zinn and Butow (1984, 1985) used southern blot analysis to describe the kinetics of budding yeast mitochondrial gene conversion events in mitotic cells. Some yeast strains carry a transposable intron within mitochondrial rDNA (ω^+) that is transferred after conjugation to a specific target site in rDNA of ω^- strains. Zinn and Butow showed that this transfer occurred after the formation of an in vivo DSB and that the insertion of the ω^+ intron also led to co-conversion of adjacent regions that was more frequent for sites near the DSB site.

A detailed analysis of homologous recombination in mitotic cells followed a few years later, with the description of the kinetics of HO endonuclease-induced *Saccharomyces* mating-type (*MAT*) switching (Connolly et al. 1988)

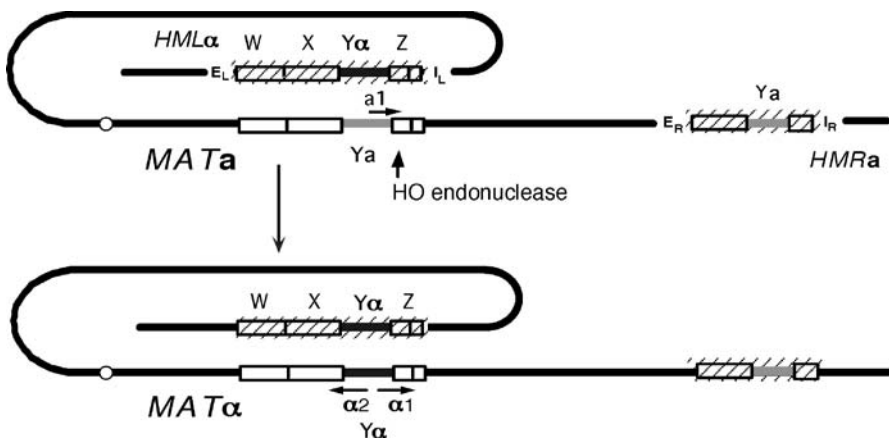


Fig. 14 *S. cerevisiae* *MAT* switching. An HO endonuclease-induced DSB in *MATa* leads to a gene conversion event using *HMLα* as the donor, resulting in the replacement of about 700 bp of *Yα* sequences by different *Yα* sequences. *MAT* and its donors share homology regions *W*, *X* and *Z* (white boxes). The two donors, *HMLα* and *HMRα*, are flanked by *E* and *I* silencer sequences (*E_L*, *I_L*, *E_R* and *I_R*) that keep the intervening sequences heterochromatic and unexpressed (indicated by hatched lines). *HMLα*, on the other side of the centromere (circle) is about 200 kb from *MAT*, whereas *HMR* is about 100 kb away, both close to their respective telomeres. An equivalent conversion of *MATα* to *MATa* occurs by recombination with the *HMRα* locus

(Fig. 14). When the *HO* gene is expressed, a DSB at the *MAT* locus leads to a replacement of the mating-type specific Y_a or Y_α sequences by gene conversion with one of two distant donors, *HML* and *HMR* (for more details about *MAT* switching, see (Haber 2002, 2007)). Detailed physical analysis of *MAT* switching was made possible by the development of a galactose-inducible *HO* gene (Jensen et al. 1983). Previous studies by Strathern et al. (1982) using the normal *HO* gene had shown that a DSB was formed in cells that were undergoing switching, but the system was not synchronized to be able to examine the progress of a single switching event. With a galactose-inducible *HO* gene Connolly et al. (1988) made the unexpected finding that *MAT* switching was a surprisingly slow process, apparently taking an hour or longer from the time of appearance of the DSB until the appearance of a product, again recognized by a different-sized restriction fragment.

The idea that DSBs were critically important for general recombination came from the demonstration that DSBs were formed transiently during budding yeast meiosis (Sun et al. 1989). Subsequent studies demonstrated that the DSBs were generated by a complex of proteins including a specialized topoisomerase, named Spo11 (Keeney et al. 1997; see S. Keeney, this SERIES). Spo11 homologs exist in all eukaryotes studied, and meiotic recombination is eliminated in the absence of those genes (reviewed by (Keeney 2001; Keeney and Neale 2006)).

8.2

Evidence of 5' to 3' Resection

Direct in vivo evidence that there was 5' to 3' resection of a eukaryotic DSB end was first provided by White and Haber (1990) who analyzed intermediates of recombination during *MAT* switching. The loss of one strand by resection could be shown on southern blots, which showed that a restriction fragment with one HO-cut end became progressively smaller and more disperse. Strand-specific probes revealed the loss of one strand, leaving the 3' ended strand intact. Moreover, on denaturing gels, using a probe for the 3'-ended strand, one could see what appeared to be a ladder of partial digestion products, because various restriction endonucleases cannot cleave sites in ssDNA.

Soon thereafter, Sun et al. (1991) showed that the DSBs made in meiosis were also resected in the same fashion. An interesting difference between mitotic and meiotic cells is that resection appears to be rather limited in meiotic cells. In meiosis, where there are many DSBs per chromosome, extensive resection between two adjacent DSBs could result in the formation of very large gaps. The limited resection also serves to restrict the length of gene conversion tracts, which prove to be much smaller in meiosis than in mitosis, even when the DSB in both situations is created by the same site-specific nuclease (Malkova et al. 2000).

8.3

Strand Invasion and 3' End Primer Extension

White and Haber made early use of PCR to show that, well after the appearance of a DSB, one could detect the expected primer extension of the 3' end that had engaged in strand invasion (White and Haber 1990). A PCR primer in *Yα* sequences unique to the *HMLα* donor could yield a PCR product with a second PCR primer distal to the *MAT* locus only after strand invasion and primer extension. This same approach was later used to reveal such intermediates in meiotic recombination.

Only more recently with the use of chromatin immunoprecipitation techniques has it been possible to detect the strand invasion step itself. After HO creates a DSB, one can detect the recombinase protein Rad51 at the *MAT* locus once the end has been resected by 5' to 3' exonucleases. Rad51 is the eukaryotic homolog of the bacterial RecA protein (for reviews see (Krogh and Symington 2004; Thacker 2005)). After a delay of about 15 or more min, ChIP reveals that Rad51 also becomes associated with the *HML* locus, 200 kb away (Sugawara et al. 2003; Wolner et al. 2003). This association represents at least the initial steps of strand invasion, prior to primer extension. In a *rad54Δ* mutant, synapsis apparently occurs but primer extension is prevented (Sugawara et al. 2003). Rad54 may play a role in the conversion of a partially base-paired paranemic (side-by-side) strand exchange joint to a fully base-paired plectonemic (interwound) structure that is necessary for PCNA recruitment and primer extension.

8.4

Physical Analysis of Double Holliday Junctions

Holliday junctions were first identified by electron microscopy by Potter and Dressler, who studied RecA-dependent branched molecules of the colicin E1 plasmid in *E. coli* (Potter and Dressler 1976). They termed these putative Holliday junctions “Chi” structures. Subsequently, Bell and Byers developed one-dimensional gel electrophoresis conditions to identify branched DNA structures from 2 μ plasmid DNA isolated from meiotic cells (Bell and Byers 1979). They confirmed that these were Chi-shaped molecules by electron microscopy². However, it was the application of the two-dimensional gel electrophoresis techniques that had enabled Brewer and Fangman (1991) to identify branched DNA molecules in the midst of DNA replication that made it possible to detect branched intermediates arising from meiotic recombination between homologous chromosomes (Bell and Byers 1983). Surveying

² Although these 2 μ structures were isolated from meiotic cells, they apparently had single—not double—Holliday junction configurations. It is possible that these crossover intermediates represent the normal site-specific FLP-mediated intermediates of 2 μ DNA (Jayaram et al. 1988), stabilized by HJ-binding proteins in meiotic cells.

meiotic DNA by electron microscopy Bell and Byers saw mostly dHJ but a significant number of apparently single HJ as well. Both Collins and Newlon (1994) and Schwacha and Kleckner (1994) used 2D electrophoresis to analyze the events at a specific loci undergoing recombination in budding yeast; they showed that there were branched molecules consistent with Holliday junctions. A more detailed analysis of events at the “*HIS4::LEU2*” hotspot showed that the recombination-dependent branched structures arising during recombination were indeed fully ligated dHJ (Schwacha and Kleckner 1994, 1995). This approach took advantage of restriction fragment length differences between the “maternal and paternal” chromosomes. When the dHJ structures were denatured and the strands separated by gel electrophoresis, only maternal and paternal lengths of DNA were found, consistent with the dHJ structure (if there had been a single HJ, then two strands should have been recombinant and two strands would be parental), but if the same structure was first treated with the RuvC HJ resolvase enzyme, then both crossover and noncrossover strands could be recovered in equal abundance. These data established that a key intermediate of the DSB repair model of Szostak et al. did indeed exist.

Recently the same approach has identified the earlier single-end strand invasion intermediate during budding yeast mitosis on two-dimensional gels (Hunter and Kleckner 2001). As expected, its kinetics of appearance precede the appearance of dHJs.

So far, physical analysis of recombination intermediates in mitotic recombination, such as those expected from HO endonuclease-induced gene conversion, has failed to see either single-end invasion or dHJ intermediates, even though HO-induced events occur in nearly all cells while meiotic DSBS are generated at $\leq 20\%$ of all chromatids. There are likely two main reasons that such an attempt has failed: first, most mitotic gene conversion events occur without crossing-over and it has been suggested that these non-crossovers arise from intermediates that do not include dHJ (about which more will be discussed below); however this concern should not apply to the strand invasion step. Second, the extent of 5' to 3' resection of the DSB ends in mitotic cells is much more extensive, likely making the branched intermediates more heterogeneous in size and hence less concentrated in a single spot on the two-dimensional gels. These intermediates may also have a shorter half-life.

8.5

Control of Crossing-Over in Meiosis by Stabilizing dHJs

A distinctive difference between meiotic and mitotic recombination is the very low percentage of crossovers accompanying gene conversions in mitotic crossovers. This is evident even when the HO endonuclease-induced DSB is created at the same site in meiotic and mitotic cells (Malkova et al. 2000). An insight into the differences between meiotic and mitotic events came from

the isolation of a large number of meiosis-specific ZMM mutations that reduced the frequency of crossovers accompanying gene conversion (Borner et al. 2004; Heyer 2004; Kunz and Schar 2004; Sym and Roeder 1994). These include deletions of several components of the synaptonemal complex (Zip1, Zip2, Zip3), the helicase Mer3, and the mismatch repair protein Mlh1, along with two homologs of the Msh2 mismatch repair protein, Msh4 and Msh5, that are not involved in mismatch repair per se. Msh4–Msh5 bind selectively to a Holliday junction, surrounding both recombining chromatids (Snowden et al. 2004). Each of the “ZMM” (Zip-Msh/Mlh-Mer3) deletions reduces the level of crossovers by about half in *Saccharomyces* and, surprisingly, the mutations are mutually epistatic (that is, multiple mutants are no more severely blocked than any single mutation). These mutations do not prevent non-crossover events and in fact the total frequency of gene conversion events is not significantly altered, suggesting that some of the intermediates initially destined to be crossovers are re-routed without ZMM to become gene conversions without exchange. In *Caenorhabditis* lack of Msh4 or Msh5 completely eliminates exchanges (Kelly et al. 2000). What remains a mystery in all eukaryotes is the identity of the HJ resolvase that acts in concert with ZMM proteins.

8.6

Identification of a HJ Resolvase

Holliday's model depended on the existence of a resolvase to generate an equal number of crossover and noncrossover alternatives. The identification of RuvC as the *E. coli* HJ resolvase was a major breakthrough (Connolly et al. 1991). RuvC cleaves with a mild sequence preference (Shah et al. 1994). The ability to branch-migrate the HJ to orient a particular sequence adjacent to the branch point is carried out by the *E. coli* RuvA and RuvB proteins, where RuvA recognizes the HJ and RuvB is a helicase that can effect branch migration (Shinagawa and Iwasaki 1995; West 1997; Yamada et al. 2002). In eukaryotes, the identification of an authentic HJ resolvase, that cleaves preferentially covalently closed, symmetric sequences, has remained elusive.

Recently, our understanding of crossover control has both been enriched and made more complicated with the discovery that the Mus81 endonuclease, with its partner Emel in *S. pombe* and Mms4 in *S. cerevisiae*, has a significant effect on meiotic, but not mitotic crossovers. In fission yeast, the absence of Mus81 nearly completely eliminates meiotic crossovers (Boddy et al. 2001; Osman et al. 2003; Smith et al. 2003). In budding yeast, the effect is less severe; loss of Mus81 has no significant effect when deleted by itself. However, loss of Mus81 or Mms4 eliminates most crossovers that were not eliminated by the “ZMM” mutants (Argueso et al. 2004; de los Santos et al. 2001, 2003). Mus81-deficient mice are fertile, suggesting that most crossovers in mice probably don't depend on Mus81 (McPherson et al. 2004).

Mus81-Eme1 will cleave—though poorly—fully ligated single HJs (no one has investigated dHJ resolution), but it is much more active on nicked, branched molecules (Boddy et al. 2001; Osman et al. 2003; Smith et al. 2003). Whitby (2005) has proposed an alternative pathway leading to crossovers in which Mus81-Eme1 cleaves an earlier, unligated intermediate (Fig. 15). Re-

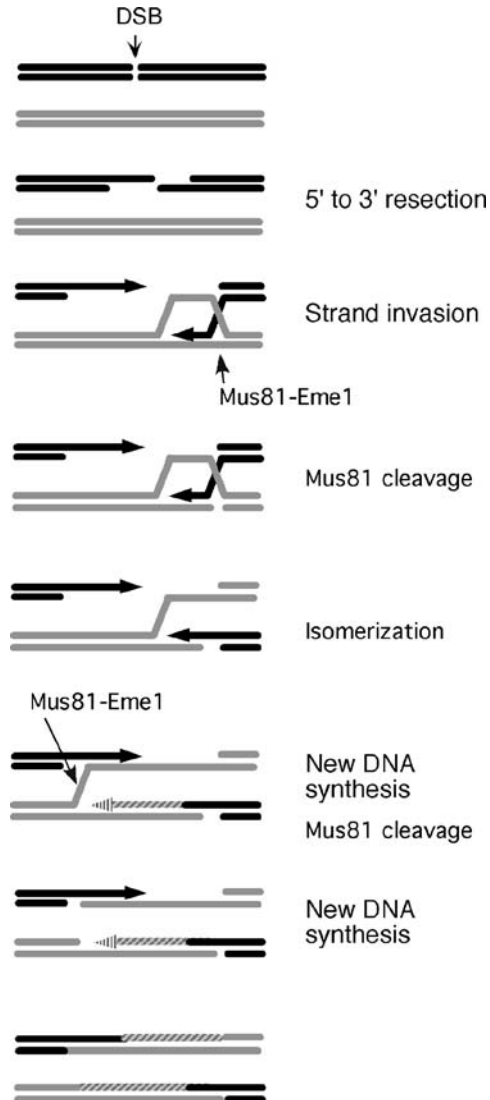


Fig. 15 A crossover-generating DSB repair model by Whitby (2005). Mus81-Eme1 preferentially cleaves nicked HJs. Cleavage of structures that resemble nicked or partial HJs results in a gene conversion event associated with crossing-over

cently Cromie et al. (2006) may have helped clarify why Mus81 has such a profound effect on meiotic recombination in *S. pombe*. Unlike budding yeast, fission yeast meiotic recombination appears to be associated with a high proportion of single HJ intermediates (see G. Cromie and G.R. Smith, this BOOK). Thus Mus81 may deal with a class of substrates (single HJ) that are less often found in budding yeast or mouse meiosis. In any case, it is clear that, at least in *S. pombe*, Mus81-Eme1 is the principle HJ resolvase.

In *Saccharomyces*, the fact that deletions of Mus81 or Mms4 affect the 25–50% of crossovers that are not affected by deletions of members of the ZMM pathway suggests that budding yeast uses at least two recombination mechanisms leading to crossing-over (Argueso et al. 2004; de los Santos et al. 2001, 2003). It is likely that the two crossover-generating pathways act on different molecular intermediates and that the intermediates attacked by Mus81 have not been visualized on 2D gels in budding yeast. These recent findings both emphasize that Mus81 may work on one class of recombination substrates and also make clear that the identity of the resolvase capable of dealing with dHJs has remained elusive. Although an enzymatic activity consistent with HJ resolution (and distinct from Mus81, which also exists in mammalian cells) has been partially purified biochemically (Constantinou et al. 2002), no gene encoding it has yet been identified. An intriguing result is that this new HJ resolvase activity is lost in the absence of two of the Rad51 paralog proteins, Rad51C and Xrcc3 (Liu et al. 2004).

9

Multiple Pathways Meiotic Recombination

In addition to two pathways yielding crossovers in budding yeast (i.e., one ZMM-dependent and one Mus81-dependent), it seems that most non-crossovers arise via another route, most likely synthesis-dependent strand annealing (SDSA), discussed below. First, the kinetics of appearance of non-crossovers precedes that of crossovers by about an hour. Moreover, the *ndt80* Δ mutation eliminates virtually all crossovers but has little or no effect on the appearance of crossovers (Allers and Lichten 2001a). In the case of *ndt80* Δ there is no significant second increase in the frequency of non-crossover events at the time normal crossovers would appear. This result suggested that by the time the proteins under the control of Ndt80 (a transcription factor) normally act the intermediates are not readily reversible to yield SDSA.

As noted above, when a purified dHJ structure is treated with RuvC, both crossover and noncrossover outcomes are recovered (Schwacha and Kleckner 1995). However, it seems that in budding yeast meiosis all of the dHJs may be resolved as crossovers, as there is not a second wave of appearance of non-crossovers at the same time that crossovers appear, as would be expected if

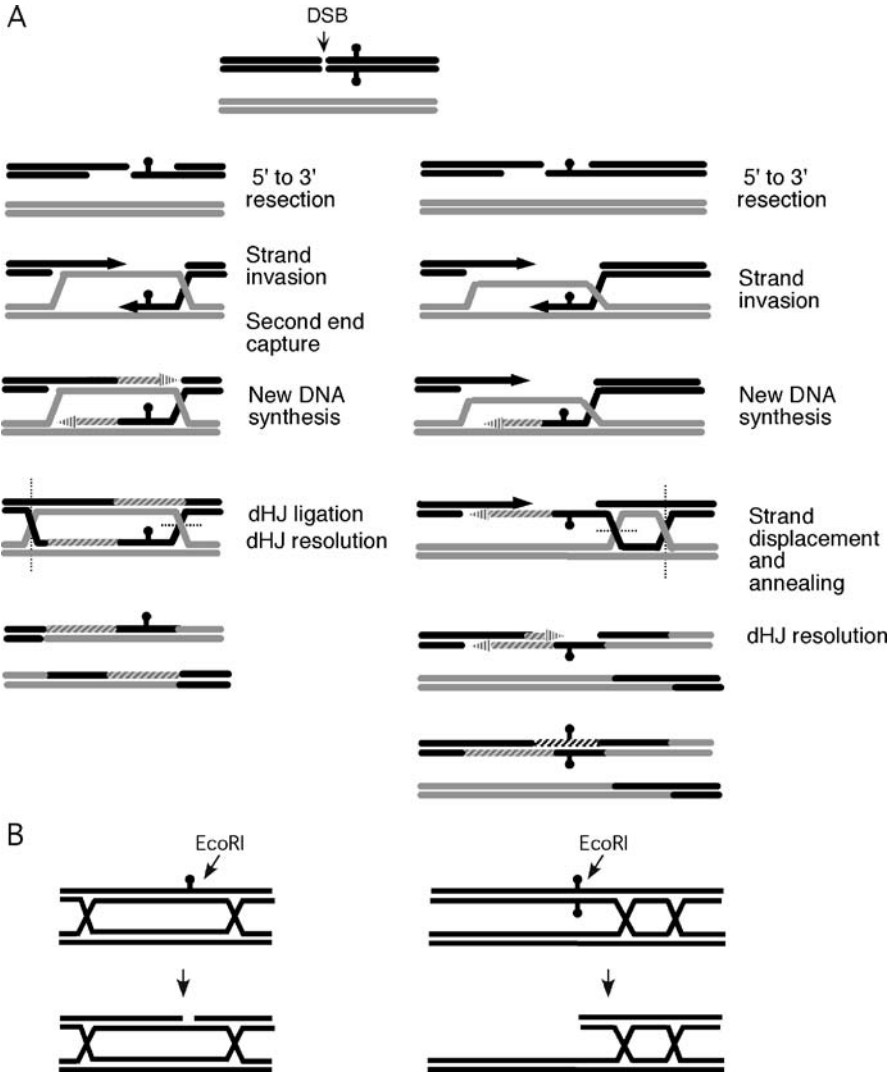


Fig. 16 Analysis of the position of heteroduplex DNA and dHJ by Allers and Lichten (2001). **A** The DSB repair pathway envisioned by the dHJ model of Szostak et al. A small insertion that creates a single-strain hairpin resistant to mismatch correction but containing an EcoRI site, is shown. **B** A modified dHJ repair mechanism in which the pair of Holliday junctions are displaced from their original location surrounding the original DSB site. In this mechanism, regions of heteroduplex can be separated from the position of crossovers. **C** Among DNA molecules identified as having dHJs by their migration after 2D gel electrophoresis are those containing an EcoRI site that can be cleaved in both in dsDNA and ssDNA. *Left*: a dHJ with heteroduplex DNA including an EcoRI site, that is seen as a nick in one strand, which is revealed when strands are separated by denaturing gel electrophoresis. *Right*: EcoRI-cleaved strands in which the position of the dHJ was displaced from surrounding the original DSB site

the dHJ intermediate could be randomly cleaved to yield both types of outcomes (Allers and Lichten 2001a). The fact that dHJ intermediates may almost always be resolved as crossovers by a mechanism different from that which produces noncrossovers can explain the finding that, in budding yeast, gene conversions accompanied by crossing-over exert interference (an inhibition of a second nearby crossover) whereas gene conversions without exchange are non-interfering (Kitani 1978; Malkova et al. 1996; Mortimer and Fogel 1974)³.

Another important finding by Allers and Lichten (2001b) concerned the locations of heteroduplex DNA and dHJ in yeast meiosis. An allele that contains a small palindromic insertion is resistant to mismatch repair when it is in heteroduplex with wild type DNA (Nag et al. 1989). The presence of heteroduplex could be confirmed in fragments containing dHJs isolated from 2D gels, but one surprise was that the dHJs did not have to span the site of the original DSB, as would be envisioned by the Szostak et al. model. Instead, it seems that there may often be branch migration and strand displacement to locate the dHJ on one side and at some distance from the site of the DSB (Fig. 16). This mechanism can account for regions of gene conversion separated by a non-converted region from the crossover site.

9.1

Meiotic Recombination in Many Organisms Depends on a Second Strand Exchange Protein

A very surprising discovery was that budding yeast, mice, *Arabidopsis* and some other organisms rely not only on Rad51 but on another (meiosis-specific) strand exchange protein, Dmc1, to carry out meiotic recombination (Bishop et al. 1992; Dresser et al. 1997; Shinohara et al. 1997; Yoshida et al. 1998). Moreover, budding yeast Dmc1 does not act primarily with the Rad51-associated proteins (the Rad51 paralogs Rad55 and Rad57 and the helicase/chromatin remodeler Rad54), but on another set of mostly meiosis-specific proteins: the Hop2-Mnd1 complex, the Mei5-Sae3 complex and on the Rad54 homolog, Tid1 (Rdh54) (Chen et al. 2004; Dresser et al. 1997; Hayase et al. 2004; Henry et al. 2006; Holzen et al. 2006; Kerzendorfer et al. 2006; Krogh and Symington 2004; Okada and Keeney 2005; Panoli et al. 2006; Tsubouchi and Roeder 2004). Moreover, in meiosis budding yeast Rad52 protein is not essential for at least some strand invasion. In budding yeast, without Dmc1 there is little recombination, and the same appears to be the case

³ It should be noted that in fission yeast, there is no evident interference (Munz 1994), whereas in *Sordaria*, Kitani (1978) found that gene conversions either with or without crossing-over exerted interference. Kitani's result was surprising and his findings strongly resisted by those working in budding yeast. His finding was made all the more surprising because crossovers not involving gene conversion (i.e., "between genes") showed interference; moreover gene conversions themselves showed a strong correlation with crossing-over (see Stahl and Foss 2007). These data suggest that there must be more than one crossover pathway in *Sordaria*. There also seem to be both interfering and noninterfering pathways in budding yeast (discussed below).

in mouse. Yet some organisms, including both *Drosophila* and *Caenorhabditis*, lack Dmc1 as well as all of its auxiliary proteins. What distinguishes these two organisms from those that use Dmc1 is that they also can effect homologous chromosome pairing and synapsis in the absence of any DSBs (Dernburg et al. 1998; McKim et al. 1998). Stahl et al. (2004) suggested that Dmc1 acts as part of a recombination machine that generates the initial strand invasion events that facilitate chromosome pairing and the formation of the synaptonemal complex, but Dmc1 appears to be required for most exchange events. Curiously, the overexpression of Rad51 or the overexpression of Rad54 will suppress the absence of Dmc1 in budding yeast meiosis (Shinohara et al. 2003), so Dmc1 is not essential for the initial recombination events that promote homolog pairing. Moreover, this suppression has an unexpected consequence: it also eliminates the normal crossover interference mechanisms that reduce the frequency of nearby crossovers. There is also an absence of interference among the crossovers that remain in the absence of the ZMM proteins. How all of these findings will fit together is not yet clear. What distinguishes Dmc1 from Rad51 and how is each recruited to DSBs?

10

Single-Strand Annealing Causes Primarily Intrachromosomal Deletions

Single-strand annealing (SSA) was originally proposed as a crossover-generating mechanism (Fig. 5A), but it seems to be most prevalent as a highly efficient intrachromosomal DSB repair mechanism (Fig. 5B). SSA appears to account for the origin of intramolecular deletions when a double-strand break is created between two directly repeated homologous sequences (Fig. 5B). Spontaneous deletions of this type were first studied by Nat Sternberg's lab (Lin et al. 1984, 1990) in DNA transformed into mammalian cells; Lin et al. suggested that long single-stranded regions could be generated by 5' to 3' exonucleases and that such regions could then anneal. An *in vitro* recombination system to study such events in *Xenopus* oocyte extracts was devised by Maryon and Carroll (1991), in which the homologous sequences were on opposite ends of a linearized DNA molecule. Maryon and Carroll provided some of the first molecular "snapshots" of the process by monitoring the intermediates of SSA on southern blots, showing 5' to 3' resection of DSB ends and the formation of heteroduplex joints. At about the same time, Rudin et al. (1989) showed similar physical evidence of SSA *in vivo* in budding yeast cells after induction of a site-specific double-strand break by the HO endonuclease. Subsequent analysis in yeast has used both HO and the I-SceI endonuclease (Fishman-Lobell et al. 1992; Plessis et al. 1992; Rudin and Haber 1988; Sugawara et al. 2000). More recently similar events between flanking Alu repeats have been induced by the I-SceI endonuclease in mammalian cells (Elliott et al. 2005). In all of these cases it is necessary

to clip off the long, 3'-ended nonhomologous tails left after strand annealing. This is accomplished in budding yeast by the nucleotide excision repair (NER) nuclease Rad1–Rad10, assisted by the Msh2–Msh3 mismatch repair (MMR) proteins, but no other NER or MMR proteins are required (Ivanov and Haber 1995; Sugawara et al. 1997). SSA in *Saccharomyces* is Rad51 and Rad54-independent, but Rad52 dependent, but it escapes even Rad52 dependence when homologies are many kb in length (Ozenberger and Roeder 1991). When the annealing homologous regions are less than 1 kb, the Rad59 protein also plays an important role (Sugawara et al. 2000).

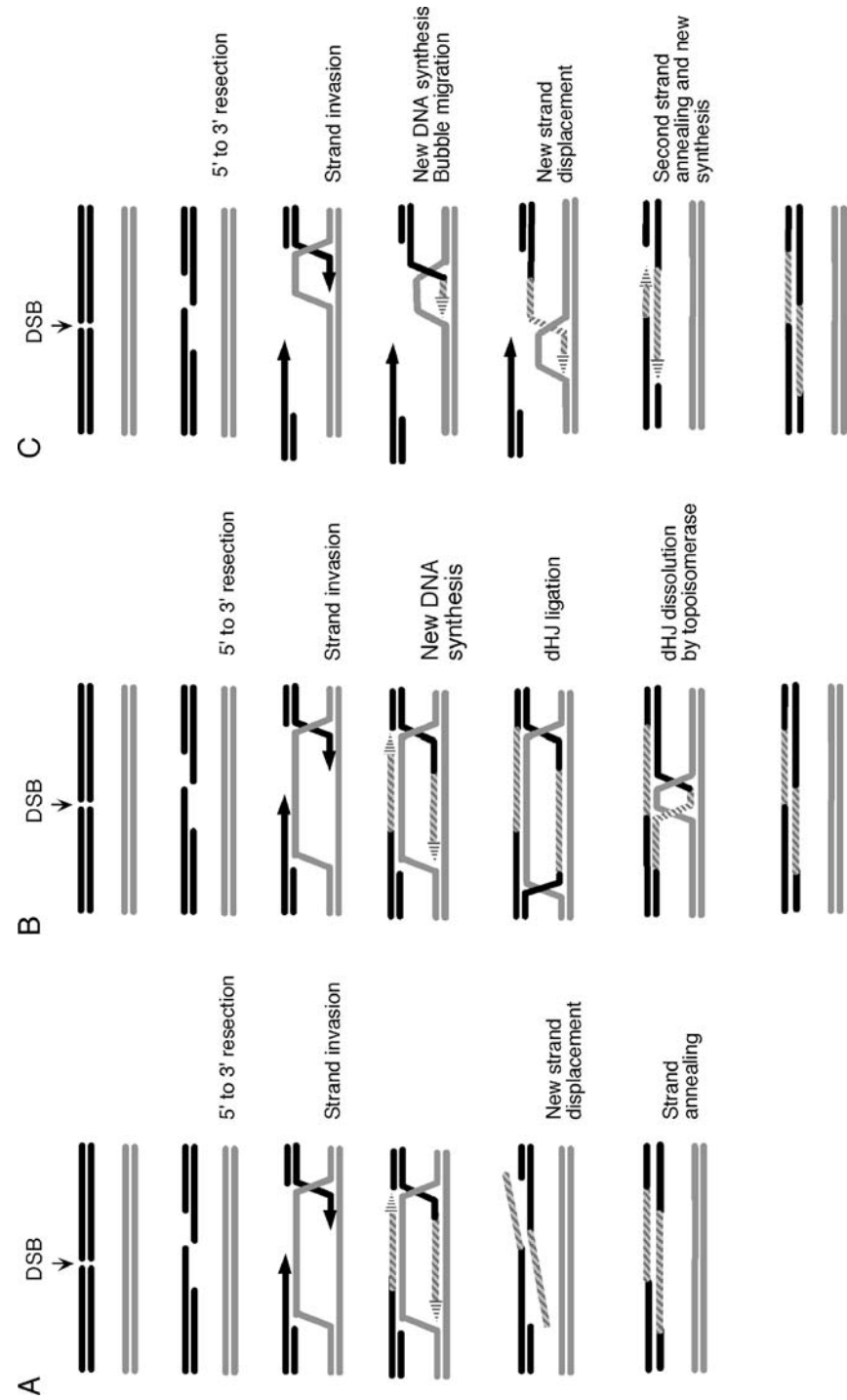
Charles Thomas' original suggestion that reciprocal crossovers could be generated by SSA was demonstrated in yeast (Haber and Leung 1996) and by Jasin's lab in mammalian cells (Richardson and Jasin 2000) using artificially duplicated sequences on different chromosomes, each adjacent to HO or I-SceI cleavage sites, to create reciprocal translocations (Fig. 5C).

It should be noted that SSA is a surprisingly vigorous process that competes with gene conversions to repair a DSB. For example, if a *MAT* locus in budding yeast is flanked with 1-kb *URA3* sequences each separated from *MAT* by several kb, 35% of the DSBs at *MAT* are repaired by SSA (deleting *MAT* and the other sequences intervening between the two *URA3* genes) even though *MAT* has evolved to undergo gene conversion at high efficiency with the *HML* and *HMR* donors (Wu et al. 1997). Resection of DSB ends appears to continue even after the Rad51-coated DSB end has located a homologous sequence (N. Sugawara and J.E. Haber unpublished).

11

Synthesis-Dependent Strand Annealing Accounts for Most Mitotic Recombination and Noncrossovers in Meiosis

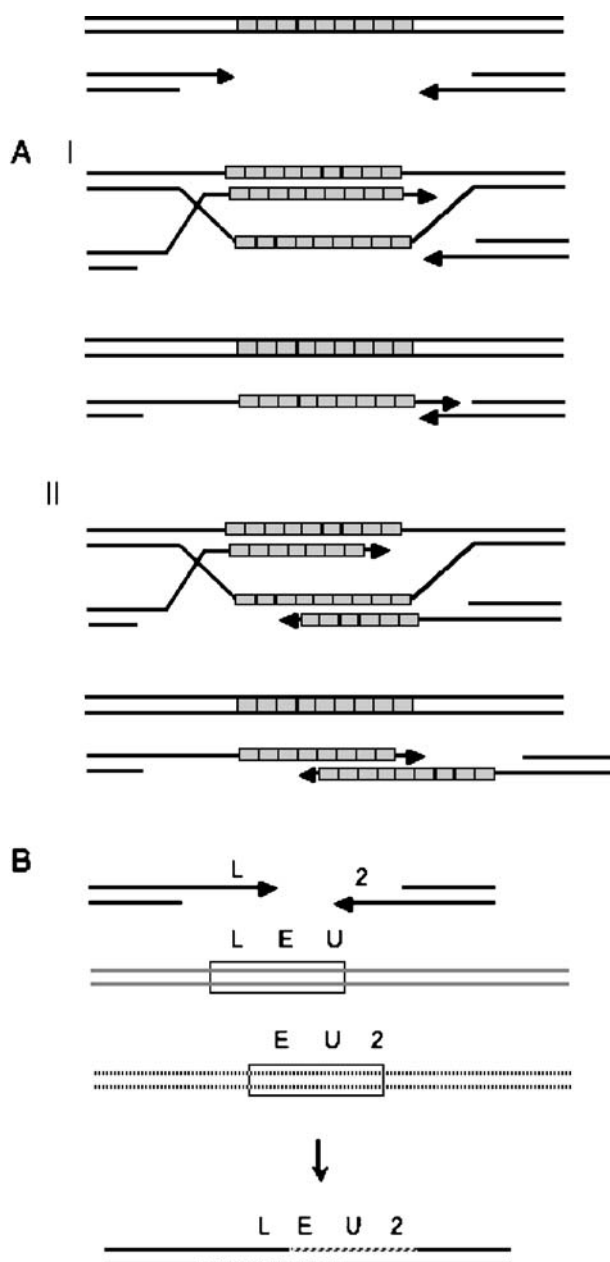
As noted before, Resnick (1976) first suggested that a mechanism involving strand invasion, primer extension, dissociation and annealing to the second resected end could account for DSB repair in the absence of crossing-over (Fig. 11A). Gloor et al. (1991) arrived independently at a similar mechanism in accounting for the repair of transposon excision-induced DSBs in *Drosophila* that occurred almost always without an associated crossing-over. As with mating-type gene switching in both budding and fission yeasts, gene conversion events induced by excision of the P-element in *Drosophila* was "directional" in that the template region remained unaltered while new sequences were "pasted in" to the recipient locus, where the excision had left a DSB. In synthesis-dependent strand annealing (SDSA), the two ends of the DSB invade a donor template and copy it; however, the replication process differs from normal replication—and from that envisioned in the dHJ model—in that the newly synthesized strands do not remain base-paired to its template. Instead, they are unwound and anneal to each other (Fig. 17A).



- ◀ **Fig. 17** Synthesis-dependent strand annealing models. **A** SDSA by means of dissolution of a double Holliday junction by means of a helicase and coupled topoisomerase, as proposed by Thaler et al. (1987) and Hastings (1988). **B** SDSA as proposed by Nassif et al. (1994). The process is similar to that described by Resnick (Fig. 10), except that there is no D-loop nicking so that the displacement of the newly synthesized strand leaves the donor template unaltered. **C** SDSA as proposed by Ferguson and Holloman (1996). The invasion of one end of the DSB promotes new DNA synthesis within a small, moving D loop “bubble,” displacing the newly synthesized strand (Formosa and Alberts 1986), which eventually can anneal with the second end of the DSB

An alternative view, leading to the same conclusion, came independently from Thaler, Stahl and Stahl (1987) and from Hastings (1988), who proposed that the dHJ intermediate of the Szostak et al. model could be unwound by topoisomerases (Fig. 17B). A subsequent modification, returning to Resnick's idea that one end would invade and, after copying, anneal to the second end was suggested by Ferguson and Holloman (1996). As first suggested by in vitro analysis of new DNA synthesis promoted by phage T4 recombination and replication proteins (Formosa and Alberts 1986), the newly synthesized DNA would be displaced, much the way RNA is synthesized from the dsDNA template, leaving the template unaltered. This displacement would continue until the second, resected end of the DSB could anneal with this new strand and initiate a second primer extension to complete DSB repair without crossing-over (Fig. 17C). The analysis of many P-element-induced gene conversions also revealed another complexity: some of the replacements of sequence at the excised locus appeared to have involved the use of more than one template (Lankenau 1995; Nassif et al. 1994). Such events were not anticipated by a dHJ model but could be accounted for by SDSA.

Pâques et al. (1996) provided a clever experiment that supported the SDSA repair mechanism by providing a template for a *Drosophila* P element-induced DSB that contained eight 375-bp repeats, so that gene conversion would require that the gap (with the 8 repeats) be copied into the recipient locus. Both in the initial experiments done in *Drosophila* and in a similar experiment done in yeast with an HO-induced DSB (Pâques et al. 1998), about half of the gap-repair events produced recipients in which there were either fewer or more than eight repeats, ranging from 1 or 2 to as many as 13 (Fig. 18A). There were almost no changes in the donor locus. This result is fully compatible with an SDSA mechanism but inconsistent with a dHJ model in which new DNA synthesis is found both at the donor and the recipient. The striking result is that still half of the recombinants have the expected 8 copies. This result could be expected if one end invaded and new DNA synthesis traversed the entire gap and then annealing took place with the other end that had not engaged in any new synthesis, but for there to be more than 8 copies it was most likely that both ends invaded independently and—after copying more than four repeats in each direction, dissociated and annealed to produce a repaired locus with 9, 10 or more repeats (Fig. 18A). Alternatively, one end



invaded, copied some repeats, dissociated and copied more before completing the repair.

Pâques et al. (1998) also created a “triparental” test to demonstrate that SDSA could occur when each end of a DSB on a plasmid was homologous

- ◀ **Fig. 18** SDSA promotes changes in copy number when repeated sequences are present in the donor template. **A** If one end initiates new synthesis and copies a region containing repeated sequences before the second end independently invades and initiates new synthesis (I), annealing will yield a repair event in which the recipient has the same number of copies as the donor. If both ends engage near-simultaneously in SDSA, then the two partially synthesized strands can anneal in a variety of alignments (II), yielding a recipient with either fewer or more copies of the repeats than the donor. **B** Triparental recombination to create an intact *LEU2* gene from two donor templates each of which is homologous only to one end of a DSB. This process requires two strand invasions, new DNA synthesis and new strand displacement. It is also possible that one end invades, copies part of the donor, displaces and invades the second template, copying the remaining sequences needed to anneal with the second DSB end

to sequences on two different templates located on different chromosomes (Fig. 18B). A complete *LEU2* gene could be created only if one end (L) could invade a template having only “LEU” and the other end (“2”) could invade a template on another chromosome carrying only “EU2”. Each single end invasion is incapable of forming a complete gene, so that there must be a noncrossover (most likely SDSA) event to construct the intact gene. The efficiency of the triparental event was about 1/40 of gene conversion of the same HO-cut sequence with an intact *LEU2* template. More recently when an analogous experiment was performed, but where all interacting sequences were inserted into chromosomes, it was found that the efficiency of such three-party gene conversion occurs about 45% as often as an interchromosomal gene conversion event between an HO-cut *LEU2* and an ectopic *LEU2* template (S. Jain and J.E. Haber, unpublished observations).

SDSA predicts that all the newly synthesized DNA should be located in the recipient locus, and indeed this appears to be the case of *MAT* gene switching in budding yeast, initiated by a DSB in G2-arrested cells (so there was no competing normal DNA replication). When nocodazole-arrested cells, having completed DNA replication, are shifted from “heavy” isotope ^{15}N ^{13}C medium to normal “light” ^{14}N ^{12}C medium at the time that HO is induced to promote *MAT* switching, all of the light DNA is found in the *MAT* locus, with the *HMR* donor left unchanged (Ira et al. 2006). A similar “conservative replication” result was previously found for *mat* gene switching in fission yeast (Arcangioli 2000), where the process occurs in S phase after a single-strand nick or fragile site is converted by replication into a broken replication fork (Arcangioli and de Lahondes 2000; Vengrova and Dalgaard 2005).

Although the experiments mentioned above demonstrate that gene conversion can often occur by SDSA, they do not rule out that some gene conversions could occur after formation of a symmetrical dHJ, as proposed by Szostak et al. Indeed, this intermediate could be “dissolved” by the combined action of a helicase and a topoisomerase so that the final outcome would be indistinguishable from the SDSA mechanism—all the newly synthesized DNA would be located at the recipient locus. In vitro evidence that fully lig-

ated dHJ could be unwound in this fashion was provided by Wu and Hickson (2003) using the human BLM (Bloom syndrome) helicase and Topoisomerase 3a. In budding yeast, evidence for such a process came from analyzing HO-induced ectopic gene conversions and the effect of the BLM homolog, Sgs1, and its associated Top3 (Ira et al. 2003). In wild-type cells about 4% of ectopic gene conversions were accompanied by crossing-over; but in *sgs1* Δ or *top3* Δ cells crossovers rose to about 12% of the total, with little or no loss of viability. These results argue that perhaps 12% of wild-type mitotic ectopic gene conversions go through a dHJ intermediate but that at least 75% of them are unwound into noncrossover outcomes.

In fact, there seem to be three helicases that act during mitotic gene conversion, each of which contributes independently to the suppression of crossing-over. Deletion of Mph1, budding yeast's homolog of the helicase domain of mammalian FANCM and archeal Hef proteins, also raises ectopic crossovers about three-fold and a *sgs1* Δ *mph1* Δ double mutant has levels of crossing-over that approach meiotic levels (> 30%), with little reduction in viability (G. Ira, pers. comm.). Mph1 may act early in the strand invasion process to channel recombination into a true SDSA pathway, though how it might do this is not known.

The third helicase, Srs2, also seems to act in SDSA (Ira et al. 2003). In vitro studies have shown that Srs2 is able to displace Rad51 from ssDNA (Krejci et al. 2003; Veaute et al. 2003) and this idea has been taken up to explain that the recruitment of Srs2 to sites of stalled replication or post-replication repair serves to ensure that Rad51 is prevented from initiating homologous recombination (Friedl et al. 2001; Ulrich 2001). Indeed the fact that many synthetically lethal double mutant combinations among *srs2* Δ , *sgs1* Δ , *rad50* Δ , *rrm3* Δ , *rad54* Δ and *mus81* Δ (among others) are suppressed by *rad51* Δ (Gangloff et al. 2000) has led to the notion that inappropriate homologous recombination leads to lethal recombination structures. What kind of Rad51-mediated events could be lethal is as yet hard to imagine, but one possibility is that single Holliday junctions are formed that cannot be resolved, at least in mitotic cells. An analogous situation has been studied in *E. coli*, where UvrD or Rep1 helicase mutants are lethal in the absence of the HJ resolvase RuvC, but this lethality is rescued by deleting RecA (Bidnenko et al. 2006).

In any case, the absence of Srs2 has a profound effect on ectopic gene conversion, though much less on allelic events. Approximately two-thirds of the mitotic gene conversions without crossing-over are lost in *srs2* Δ cells, and no alternative product is seen to account for their absence on a southern blot (Ira et al. 2003). As a consequence, the proportion of crossovers among the successful recombination events goes up about 3-fold. An independent indication that most SDSA events are eliminated in *srs2* Δ comes from the finding that the kinetics of appearance of noncrossovers is now coincident with crossovers, whereas in wild-type and in *sgs1* Δ cells noncrossovers

appear about 30 min earlier than crossovers (Ira et al. 2003). The kinetic difference argues that most noncrossovers arise from a separate pathway and not by simple alternative resolution of a common dHJ structure.

It is hard to understand how Srs2 would selectively affect noncrossovers and why its absence would be so often lethal if its role were simply in regulating the ability of Rad51 to form a filament on ssDNA prior to strand invasion. If anything, one might have imagined that longer Rad51 filaments in the absence of Srs2 would favor the stable dHJ intermediate rather than SDSA, where the newly synthesized strand must be displaced from its template. Perhaps Srs2 acts to prevent Rad51 from binding to the displaced template strand in the D-loop, but again, should not this binding facilitate strand pairing with its complement after the invading strand is displaced? Srs2 may act at the later step of displacing the newly synthesized strand from its template.

Surprisingly, none of the helicases discussed above plays a major role in meiotic recombination in budding yeast. The lack of effect of these helicases is understandable if they are all evolved to prevent a high level of crossing-over and hence reduce loss of heterozygosity in mitotic cells. In meiosis, deleting Sgs1 has very little effect on crossovers in otherwise wild-type cells, but it does dramatically increase the proportion of exchanges in cells lacking one of the ZMM proteins (Jessop et al. 2006; Oh et al. 2007). In meiosis, the evidence suggests that most dHJ intermediates are recovered as crossovers; it has been suggested that the ZMM proteins normally stabilize dHJs and prevent access of Sgs1-Top3; when this protection is disrupted, Sgs1-Top3 could act to unwind dHJs into gene conversions without exchange. Curiously, deleting *S. pombe*'s BLM homolog, Rqh1, has a profound effect in lowering crossovers (Ponticelli and Smith 1989).

12

Evolution of Gene Conversion Models in the Present

The evolution of recombination models can be thought of as an example of punctuated equilibrium (Eldredge and Gould 1997). After periods of stasis, a new model emerges that dominates the scene for a period of time until sufficient objections arise to force a new view of the molecular events, but while one is living in a time when most things seem settled, it is virtually impossible to see the design of the most robust solution that will emerge. The best we can do is identify some observations that are not easily accommodated into our present picture—even one in which there are at least two crossover-generating and two noncrossover-generating gene conversion pathways. Much confusion surrounds the role of mismatch repair, especially in affecting meiotic recombination.

It has previously been shown that in budding yeast, 6 : 2 or 2 : 6 gene conversions accompanied by crossing-over show positive interference on the position

of an adjacent crossover. Interference is seen even when crossing-over occurs with no obvious gene conversion within the interval, But Stahl's lab (F. Stahl, pers. comm.) now reports that crossovers in which a marker exhibits 5 : 3 or 3 : 5 segregation are noninterfering. These results suggest that there are two distinct crossover-generating pathways that differ in how heteroduplex is mismatch corrected. Moreover, the absence of Msh4 (which is a ZMM protein and which has no evident role in mismatch repair despite its homology to Msh2) reduces the proportion of 6 : 2/2 : 6 events and increases the frequency of 2 : 2, without affecting the proportion of 5 : 3/3 : 5 outcomes. What the relationship between mismatch repair and the channeling of recombination intermediates into crossover or noncrossover pathways is unclear.

These findings are reminiscent of studies from Jean-Luc Rossignol's analysis of meiotic recombination in the *b2* locus of *Ascomolus*. As mentioned earlier, the presence of a large heterology in the middle of the gene blocked formation of Ab4 : 4 tetrads without affecting the levels of 5 : 3 or 3 : 5 of poorly corrected mutations lying distal to the large insertion/deletion. This was taken as evidence that branch migration could not go past a heterology. Consistent with the notion of a blocked HJ (or dHJ), the heterology also causes a large increase in crossovers within the gene, but curiously, the frequency of crossovers was strongly affected by the presence of a single-bp heterology far upstream (Langin et al. 1988a,b). When that heterology was well-corrected by mismatch repair, the level of crossovers was high; when the upstream marker was poorly repaired, the incidence of crossovers was markedly reduced. These data suggest that the strand-nicking and repair events associated with mismatch repair somehow are linked to the process either of creating a stable crossover intermediate or of resolving that intermediate as a crossover.

A second problem involving mismatch repair concerns the location of heteroduplex DNA and its relationship to asymmetries that may be produced at the initiation of meiotic recombination. The dHJ model predicts there should be equivalent regions on each side, assuming resection is equivalent on both sides. However, Keeney has recently shown that the removal of Spo11 from DSB ends leaves strikingly asymmetric regions of ssDNA (Neale et al. 2005), though how this would affect the final outcome is not yet clear. Heteroduplex DNA should be located on two different chromatids when there is no crossover and adjacent when there has been a crossover. If the parent that experienced the DSB had a and b alleles on either side, they should be in an a/+ and +/b arrangement. Recent studies by Hoffmann et al. (2005) and by Stahl's lab (F.W. Stahl, pers. comm.) have revealed a profound difference in apparent outcome when a heteroduplex adjacent to the DSB could not be mismatch repaired. When heteroduplex DNA on both sides of a DSB could be repaired efficiently 90% of the time there was a conversion event on both side of the DSB (that is, only 10% appeared "one-sided"), both in crossover and noncrossover situations, but when the marker on one side is poorly re-

paired or when the cells lack mismatch repair, 50% of the events appeared to be "one-sided." Hoffman et al. interpreted these differences as evidence that heteroduplex could be restored to the genotype of the resident (non-invading) strand by a mechanism not depending on mismatch repair. Stahl and colleagues suggest that mismatch correction can occur in two different points in recombination and that the outcomes that are recovered when there is a poorly repaired allele reflect the absence of rapid correction of heteroduplex repair often very soon after strand invasion, a type of repair demonstrated by physical analysis of early events in mitotic cells undergoing *MAT* switching (Haber et al. 1993).

There is still another problem that emerges from these studies. If heteroduplex DNA forms on each side of the DSB, the dHJ model suggests when a crossover occurs, there should be 2 chromatids containing heteroduplex, one with a/+ and one with +/b, but in fact, one often finds a/+ and +/b on one chromatid, yet still associated with a crossover (Hoffmann and Borts 2005). If one assumes that the DSB actually did occur between the two markers, this arrangement of heteroduplex DNA can occur only if the initial strand invasion structure is altered by branch migration, either of a dHJ (Hoffmann and Borts 2005) or in SDSA, similar to the process suggested by Allers and Lichten (2001b).

Of course, the state of heteroduplex can only be seen if the markers are poorly repaired. When markers can be repaired, there is a further concern: Borts et al. (1987, 1990) showed that adding mismatches to a region to more precisely locate the length of a gene conversion tract and the site of crossing-over had the disturbing effect of altering the outcome. Even with a density of markers as low as 1/kb, apparently independent mismatch repair events at adjacent sites provoked the creation of resection-induced secondary recombination events that altered the outcome. These secondary breaks could involve recombination with a third chromatid and were revealed by provoking single-strand annealing between fortuitously placed repeated sequences.

MAT switching has also left us with at least one outcome that is not easy to square with our current picture. Strand invasion after HO cleavage occurs first on the side where there is perfect homology between the DSB and the donor on the right side of the DSB (i.e., the *MAT*-Z region shown in Fig. 14). Invasion is followed by primer extension and this occurs much earlier than the opposite side of the DSB, where more extensive resection and removal of nonhomologous *Ya* or *Yα* sequences must occur before primer extension can occur. If there is a single bp *stk* mutation in *MAT*-Z, this is rapidly corrected before the time that primer extension can be seen (that is, quite soon after strand invasion), but in 23% of wild-type cells and in 59% of *pms1* cells lacking mismatch repair, *MAT* switching produces a cell that gives rise to a sectorized colony, suggesting that the mismatch was not repaired and was in heteroduplex with a copy of the donor sequence (Ray et al. 1991). Without *Pms1* there was also a big increase in the proportion of cells in which both

DNA strands carried the *stk* mutation, which is the expectation of a standard SDSA mechanism, but how would the sectorized colonies arise if the second strand is copied from the displaced first strand still carrying its mutation? These data suggest that the copying of the second strand often uses the donor as its template, a result that would be more compatible with this gene conversion without crossing-over resulting from unwinding of a dHJ.

13

Another Major Source of Creative Thinking: Nonreciprocal Recombination in Phage λ

A major influence on thinking about recombination was the seminal book by Frank Stahl, *Recombination: Thinking about it in phage and fungi* (Stahl 1979). Stahl's lab had focused on an 8-bp *cis*-acting recombination enhancing element called Chi (χ) (Lam et al. 1974; Stahl et al. 1980, 1983) which proved to have the sequence GCTGGTGG (Smith et al. 1981). Chi was dominant (only one partner needed to have Chi) and acted directionally, downstream from the open end of the linearized phage λ (when λ becomes linear, one end is protected from attack by a protein complex). The DSB end is attacked by the *E. coli* RecBCD complex that cleaves frequently in strand ending 3' and infrequently on the strand ending 5' (Amundsen et al. 1990). The movement of RecBCD could either be processive, with frequent cleavages or by unwinding, with less frequent cleavages (Taylor et al. 1985). This degradation process continues until the enzyme reaches Chi, which is bound by RecBCD and dramatically affects its activity, so that it moves more slowly and now cleaves only in the 5' to 3' direction, leaving a long 3' ended ssDNA that ends near Chi (Dixon and Kowalczykowski 1993). The ssDNA Chi sequence itself appears to be a weak preferential loading site for the RecA recombinase (Anderson and Kowalczykowski 1997; Churchill et al. 1999), which can then promote recombination with a circular λ molecule. Thus recombination is promoted downstream of Chi. Phage λ does not naturally have a Chi site—the ones studied were obtained by mutations that enhanced phage recombination; but *E. coli* has many such sequences which seem designed to “dampen” the activity of the voracious RecBCD nuclease. Surprisingly, although this remarkable system is highly conserved among bacteria, it does not seem to have been preserved in eukaryotes. Nevertheless, although there do not seem to be Chi-equivalent sites in eukaryotes, the intensive study of Chi, and the biochemistry of RecBCD, provided a conceptual basis for thinking about DSB end-processing by nucleases. Pioneering studies by Gerry Smith's lab (Smith 2001) were followed by both single-molecule analysis of RecBCD action (Spies et al. 2003) and an incredibly informative structure from X-ray crystallography that could explain many of the remarkable properties of the RecBCD enzyme (Singleton et al. 2004).

The idea that RecBCD-like enzymes could unwind DSB ends with little or no degradation stimulated the idea that single-stranded ends ending either 5' or 3' might be effective in promoting recombination. "Split end" models of recombination have been proposed by Rosenberg and Hastings (1991), based in part on Rosenberg's findings of recombination outcomes that might best be explained if strands of either polarity could be assimilated in recombination intermediates (Hagemann and Rosenberg 1991).

As discussed more fully below, this one-DSB-end recombination system produces non-reciprocal recombination products. The most recent data suggest that these outcomes arise not by a crossing-over but by recombination-dependent DNA replication.

14

Re-Emergence of Old Ideas in New Guises: Break-Induced Replication

Over the past decade, increasing attention has been paid to another major mechanism of homologous recombination. One of the earliest molecular ideas about recombination was that crossing-over involved a break and copy mechanism (see Fig. 1) (Meselson and Weigle 1960). A more molecular version of this mechanism was proposed by Anne Skalka as "a replicator's view of recombination (and repair)" to explain phage λ recombination (Skalka 1974) and a similar proposal of recombination-dependent DNA replication by Mosig (1987) could account for late recombination events in phage T4 infection. Kogoma provided evidence for recombination-dependent DNA replication in the replication of *E. coli* chromosomes lacking their normal origin of replication, by so-called stable DNA synthesis (Kogoma 1996, 1997).

This notion of recombination-dependent DNA replication did not penetrate the consciousness of people studying eukaryotic recombination until attention was focused on three key biological problems. The first is how cells re-start DNA replication at a broken replication fork, which essentially consists of a template and a one-ended, partially replicated sister chromatid (Haber 1999; Michel 2000; Michel et al. 2004). The second is how "ends-out" linear transforming DNA was integrated into a genome (Dabert and Smith 1997; Smith 2001)⁴. The third is how eukaryotic telomeres are maintained in yeast and in some human cancer cells in the absence of the telomerase enzyme (Dunham et al. 2000; Henson et al. 2005; Le et al. 1999; Lundblad and Blackburn 1993). In each case, a coupled recombination/replication

⁴ "Ends-in" and "ends-out" orientations were defined for linearized DNA segments depending on the orientation of sequences at the two ends that share homology with a template chromosome. The "ends-in" orientation is equivalent to a DSB in a circular plasmid in which the regions on either side of the DSB are homologous to a template, the two ends of the DSB face each other and could be repaired either by end-joining or by gene conversion (e.g. Figure 12). In "ends-out" the two ends are facing away from each other and would be expected to result in a gene replacement, for example if there were crossing-over at each end of the linear fragment (Rothstein 1983).

event provided a satisfactory explanation. A D-loop intermediate created by recombination-dependent strand invasion would be followed by the establishment of a unidirectional replication fork that could proceed to the end of a template (Fig. 19). This process is now often termed break-induced replication (BIR).

In *E. coli*, there is now substantial evidence that many types of recombination involve extensive break-copy events. The idea that, in *E. coli*, a fragment of transforming, conjugative or transduced DNA would have two independent

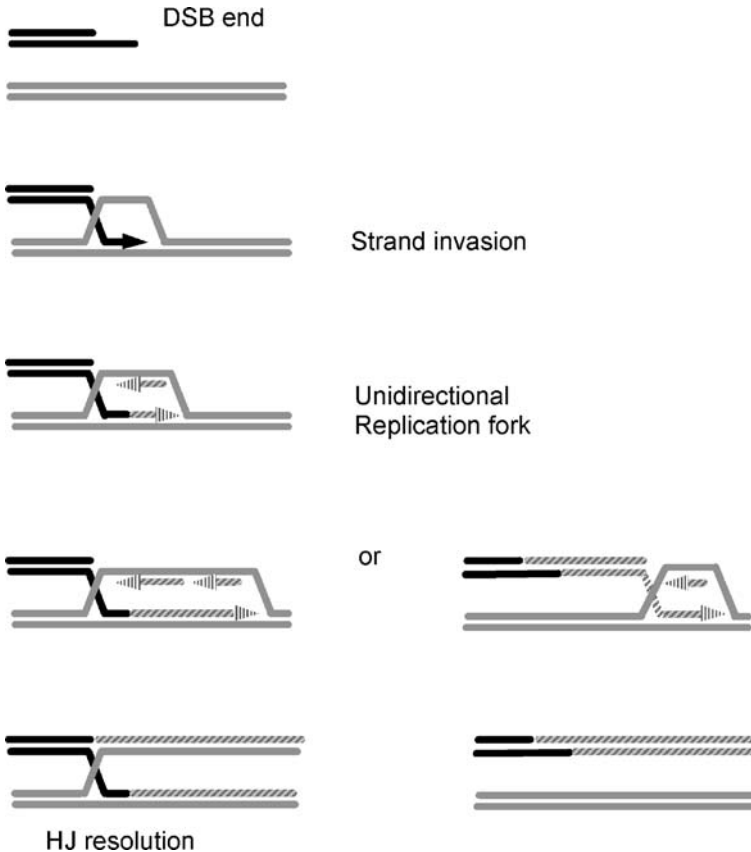


Fig. 19 Break-induced replication. In some cases, only one end of a DSB shares extensive homology with a template, for example at an eroded telomere or at a stalled and broken replication fork. Resection of the end and strand invasion creates a D-loop that can be converted to a unidirectional replication fork that can proceed to the end of the template or until it collides with an oncoming replication fork. The fate of the newly synthesized strands is unknown; they could remain semi-conservatively associated with their templates (in which case a HJ at the point of invasion needs to be resolved) or branch migration could yield one entirely “old” template and an entirely “new” BIR product

strand invasions, each establishing a replication fork to copy the remaining 5 Mb of the circular chromosome (Dabert and Smith 1997) has received strong experimental support in the studies of phage λ recombination, where such break-copy replication has been documented (Kuzminov and Stahl 1999; Motamedi et al. 1999).

In budding yeast, nonreciprocal translocations, using dispersed homologous sequences as points of strand invasion, have been documented in a number of studies. Moreover, telomere maintenance in the absence of telomerase appears to be accounted for in this way. In fact, there seem to be two different Rad52-dependent telomere maintenance pathways, one requiring Rad51 and the other requiring Rad59 and MRX proteins (Le et al. 1999; Lundblad and Blackburn 1993; Teng et al. 2000; Teng and Zakian 1999). The Rad51-dependent pathway uses larger regions of subtelomeric homology, whereas the Rad59 pathway leads to amplification of the irregular TG₁₋₃ telomere sequences themselves, possibly by a rolling circle mechanism. BIR in yeast was first proposed by Voelkel-Meiman and Roeder (1990), based on nonreciprocal gene conversion events that extended 100 kb down a chromosome arm, apparently to the end. Using transformation, Morrow et al. (1997) demonstrated that recombination-dependent DNA replication in *S. cerevisiae* could copy as much as 400 kb; more recent work would place the lower limit as at least 1 Mb (B. Llorente, pers. comm.). A more detailed analysis of BIR has been accomplished by using HO endonuclease to create a single DSB under circumstances where only one end of the DSB shares homology with a template chromosome (Bosco and Haber 1998; Davis and Symington 2004; Malkova et al. 1996, 2005). In a diploid version of the system, where an HO cut is made close to the end of a truncated chromosome, there are also two Rad52-dependent outcomes that produce diploids with two intact chromosomes, homozygous for all the markers distal to the point of the DSB. One pathway is dependent on Rad51, Rad54, Rad55 and Rad57 and the other is dependent on Rad59, the MRX proteins and Rdh54/Tid1 (Malkova et al. 1996, 2005; Signon et al. 2001). The Rad51-independent pathway frequently results in nonreciprocal translocations using dispersed Ty retrotransposon sequences as the initiating homology (Malkova et al. 2001; VanHulle et al. 2007).

The Rad51-dependent pathway is efficient, but in competition with gene conversion when there is homology on both sides of the DSB, BIR is quite rare. One explanation for the lower efficiency is that BIR is delayed for several hours relative to gene conversion (Malkova et al. 2005). The delay may reflect the slowness of establishing a replication fork after the initial strand invasion step. Recently it has been shown that BIR, but not gene conversion, requires the lagging-strand synthesis primase-Pol α proteins and also the nonessential Pol32 subunit of Pol δ . Pol32 is also required for both types of telomere maintenance without telomerase (Lydeard et al. 2007). A striking feature of BIR is that the copying process is—at the beginning—much less processive than normal DNA replication. When a broken end is confronted with more

than one possible template, there are frequent template switches, but these are confined to the first several kb of new DNA synthesis; after that, the process becomes confined to one template (Smith et al. 2007). A possible explanation for this finding is that the initial new synthesis of BIR requires Pol α -primase and Pol δ , but not Pol ϵ ; however Pol ϵ is required to complete elongation (Lydeard et al. 2007).

Both logic and ChIP analysis suggest that BIR begins with resection of a DSB end, recruitment of Rad51, and strand invasion, just as in gene conversion. What then occurs to delay primer extension and establishment of the replication fork is not yet known. It is also not clear where the newly synthesized strands end up (Fig. 19), as they could either be in a semi-conservative or conservative arrangement, depending on whether there is resolution or branch migration of a single Holliday junction that is imagined to be formed at the strand invasion step.

The intimate relationship of recombination and replication is also emphasized by the phenotype of vertebrate cells depleted of Rad51. Cells rapidly die with the accumulation of chromatid-type breaks, that is where there is one broken sister chromatid and an adjacent intact sister chromatid (Sonoda et al. 1998). Normally, these frequent breaks would be repaired by gene conversion or BIR. Recombination is as essential for life as the replication process itself and our understanding of the different mechanisms of homologous recombination and what happens when repair is compromised will aid in understanding the origins of genome instability that underlie many human diseases. In the 40+ years since Holliday set down his molecular model of recombination, we have made remarkable progress in defining the process(es) in great detail, but as we survey what we now know we realize that from a more distant future vantage point it will be clear that we missed several wonderful features, just over the horizon.

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Searching for Homology by Filaments of RecA-Like Proteins

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Abstract The recombinase proteins of the RecA family perform tasks that are essential for cell survival and for the maintenance of genetic diversity. They are able to rearrange genes in new combinations and to repair DNA double-strand breaks in an almost error-free fashion. Their function in homologous recombination is performed in an original way that has no equivalent in the DNA processing machinery: They form long helical filaments on a target DNA, capable of recognizing homologous DNA sequences in the genome and of exchanging DNA strands. How the DNA sequences are recognized during this process and how the DNA strands are exchanged remain matters of investigation. This chapter reviews the information that has been accumulated on recognition and strand exchange, together with the models that aim at organizing this data, viewed at different levels: that of the nucleus, the molecule, or the atom. Altogether, a picture begins to emerge on a multiscale dimension, which presents the search for homology as a complex process with important dynamic components.

Abbreviations

HR	homologous recombination
dsDNA	double-stranded DNA
ssDNA	single stranded DNA
EM	electron microscopy
FRET	fluorescence resonance energy transfer
ScRad51	Rad51 from <i>Saccharomyces cerevisiae</i>
MvRadA	RadA from <i>Methanococcus voltae</i>
EcRecA	RecA from <i>Escherichia coli</i>
MtRecA	RecA from <i>Mycobacterium tuberculosis</i>

1

RecA-Like Proteins and Homologous Recombination

1.1

The Universal Function of Homologous Recombination

Homologous recombination (HR) is universally spread among all life species. Its function is dual. It is responsible for gene crossovers associated with genome rearrangement during meiosis in eukaryotes, and for genome incor-

poration during conjugation and transduction in prokaryotes. This function is essential for the creation of genome diversity and for the maintenance and adaptation of the species. In eukaryotes, it has also been associated with chromosome segregation during meiosis (Campbell and Davis 1999b; Shinohara and Shinohara 2004; Li and Ma 2006). As a second function, equally essential, homologous recombination constitutes one of the main routes for DNA double-strand break repair. As such, it is tightly associated with replication, which generates this type of dramatic damage when encountering single strand breaks (Cox 1999; Kuzminov 2001). In contrast to non-homologous DNA repair processes like end-joining repair, HR allows reconstitution of the damaged DNA sequence without any loss of information. These essential functions make the understanding of the mechanism of homologous recombination a major issue for the development of anticancer strategies or gene therapies.

1.2

Nucleoprotein Filaments, the Active Form of Recombinases

At the heart of the recombination process is the alignment and exchange of two DNA strands of similar sequences, provided by a duplex DNA (dsDNA) and by a single-stranded DNA (ssDNA)¹. The ssDNA results from the processing of the target DNA to be exchanged or repaired, and the dsDNA is searched for homology within the genome. It is most readily accessible in the sister chromatid in eukaryotes, but the search is directed towards non-sister homologs during meiosis.

The sequence recognition and strand exchange process is catalyzed by a nucleoprotein filament, built on ssDNA by cooperative assembly of monomers from a single protein species, RecA for prokaryotes (Fig. 1), or the partial RecA homologs Rad51 and Dmc1 for eukaryotes. These proteins form a right-handed helix around the DNA. Other members of the HR protein family have been found to interact with DNA in a filamentous form, like archeal Rada (Woods and Smith 1997; Yang et al. 2001) or bacteriophage T4 UvsX (Formosa and Alberts 1986). Most of them can also assemble as rings, and Rada has been found to bind DNA both as a filament in the presence of ADP or ATP and as an octameric ring in the absence of cofactor (Yang et al. 2001). Dmc1 protein, specifically involved in meiosis in yeast and higher eukaryotes, was previously thought to interact with DNA as an array of stacked protein rings (Masson et al. 1999), but recent data indicate that this protein also binds ssDNA as a helical filament active for strand exchange (Sehom et al. 2004; Wyman and Kanaar 2004), with a stoichiometry of Dmc1/DNA association estimated to be three nucleotides per Dmc1 subunit (Li et al. 1997).

¹ The process of homologous recombination necessarily initiates on a DNA single strand. In vivo, the reaction can be extended to double-stranded DNA, frequently giving way to four-stranded reactions, and finally producing Holliday junctions.

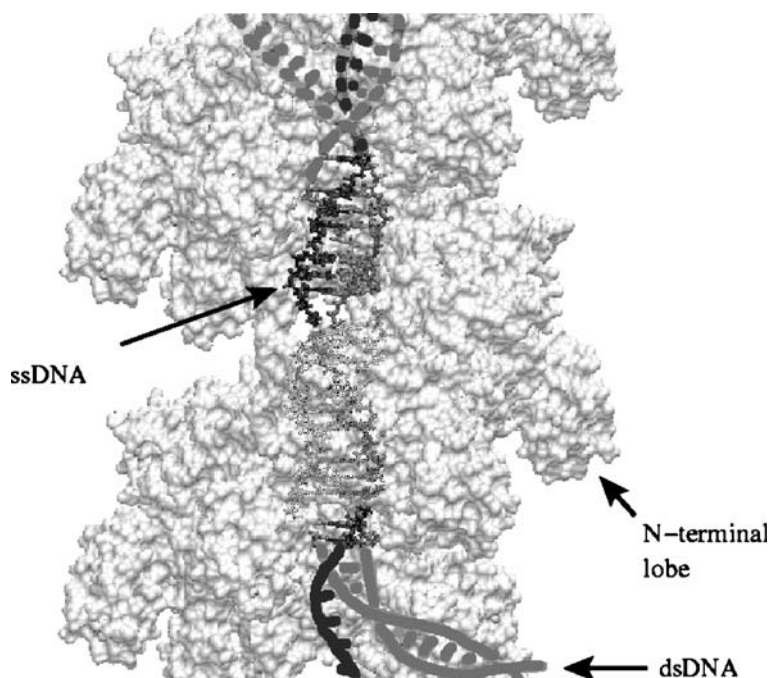


Fig. 1 Schematic representation of the strand exchange reaction taking place within a RecA nucleofilament. The view figures three RecA subunits, represented in *light gray* and in surface mode (VMD, Humphrey et al. 1996). Their coordinates are provided from the crystal structure of the RecA filament (Story et al. 1992). The positions of three DNA strands incorporated in the filament are indicated: *dark gray* for the ssDNA on which the filament initially forms; *light gray* for the incorporated dsDNA; and *dotted lines* for the DNA parts that are hidden by the protein. The two strands of the dsDNA are initially paired upon introduction (*bottom*). They are intertwined within the filament and they separate after strand exchange (*top*). The exposed part of the intertwined three strands is represented in atomic representation in an R-form triple helix, proposed to transiently form as a result of strand exchange (see Sect. 5.2)

The proteins of the RecA family share a homologous ATP-binding core that has been remarkably conserved during evolution. This core is widely found in many other proteins, including the F1-ATPase as well as proteins involved in all aspects of DNA metabolism (Aravind et al. 1999), but also in proteins that do not interact with DNA. However, the recombinase proteins from eukaryotes and prokaryotes completely differ with respect to their C-terminal and N-terminal domains in terms of both sequence and structure. The C-terminal domain of RecA is absent in its eukaryote homologs. Its N-terminal domain is composed of a single helix, while in Rad51, Dmc1, and Rada this domain incorporates HhH motifs that are known to bind DNA (Doherty et al. 1996; Aravind et al. 1999). Interestingly, the C-terminal domain of RecA and the N-terminal domains of Rad51/RadA occupy an analogous

position in low resolution structures reconstructed from electron microscopy (EM) as a lobe observed at the periphery of the filament (Yu et al. 2001)². In the case of RadA and yeast Rad51, the large amplitude mobility of this lobe has been shown to play a crucial role in activating the nucleoprotein filament ATPase and strand exchange activities by contacting the core domain of neighboring subunits (Galkin et al. 2006). In the same way, a mutant of Dmc1 protein that lacks the flexible N-terminal 81 amino acids does not bind any more DNA (Kinebuchi et al. 2005). Another characteristic common to all recombinase filaments is the presence of two flexible loops situated on their internal surface, L1 (~eight amino acids) and L2 (~20 amino acids)³ with, however, non-conserved sequences. Globally, it can be said that the nucleofilaments of different species share striking similarities regarding their helical geometry, with a little more than six subunits per turn⁴ and with comparable pitch values, typically between 80 and 110 Å (Egelman 2001). All of them have been characterized under two forms, depending on whether the cofactor is ATP (extended form) or ADP (compressed form) (Fig. 2), which are both represented among the crystal structures of recombinase filaments⁵.

Within these filaments, the constitutive as well as the incorporated DNAs are centered on the helix axis. Once it is incorporated, the dsDNA is forced to adopt a stretched and unwound conformation so that it is phased with the filament, with a remarkably conserved stretching factor of 50% with respect to canonical B-DNA (Egelman 2001). The unwinding factor is about 40% and each recombinase subunit covers three DNA nucleotides. These common features have led to several propositions for a particular role of DNA deformations in the mechanism of recognition and strand exchange, which will be developed in Sects 4.2 and 5.

The recombinase nucleofilaments also present common characteristics in their strand exchange activity. These include a phase of initiation on short DNA stretches (hundreds of base pairs), followed by the propagation of strand exchange to DNA sections several kilobases long, and finally by the release of the displaced strand. In contrast to the initiation phase, which does not require an additional energy supply (Kowalczykowski and Krupp 1995), the propagation phase and the release of the displaced strand require ATP hydrolysis (Rosselli and Stasiak 1990).

² The similarity of the lobe position, viewed at low resolution in the RecA and Rad51 filaments, is only observed when these two filaments have opposite polarities, which means that their ATP-binding core domains are positioned in opposite directions.

³ The structures of loops L1 and L2 could only be determined in the crystal structure of MvRadA, PDB code 1XU4 (Wu et al. 2005), and separately in two forms of MtRecA, 1MO5 for L1 and 1MO4 for L2 (Datta et al. 2003).

⁴ Note that filaments of *C. Elegans* Rad51 with about eight subunits per turn have recently been characterized (Petalcorin et al. 2007).

⁵ Extended forms: MvRadA, PDB code 1XU4 (Wu et al. 2005) and ScRad51, code 1SZP (Conway et al. 2004). Compressed forms: EcRecA, 2REB (Story and Steitz 1992), 1U94 (Xing and Bell 2004), and MtRecA, 1MO3 to 1MO6 (Datta et al. 2003).

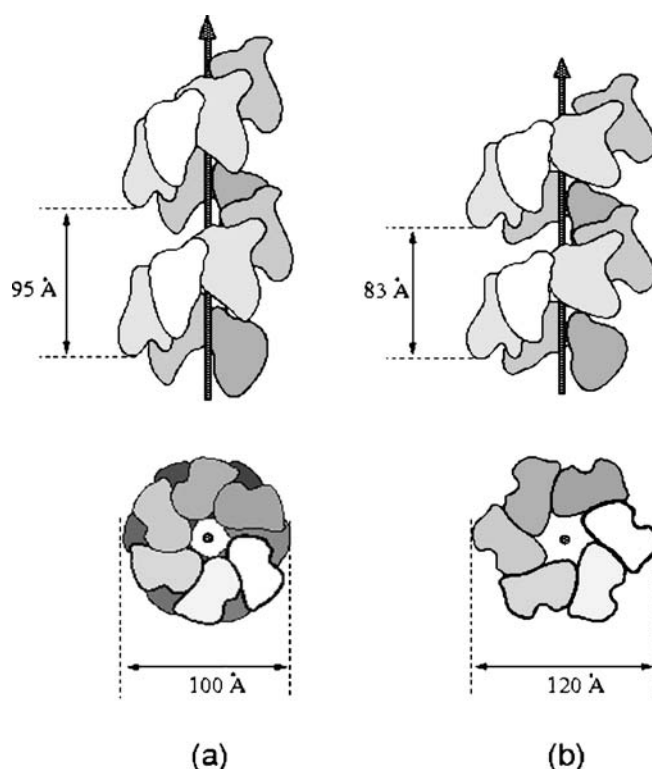


Fig. 2 Schematic representation of **a** the stretched form and **b** the compressed form of the RecA filament. The DNA is not represented. The structure in **b** is taken from the crystal structure of Story et al. (1992) (RecA/ADP; PDB entry 2REB). The *top* representations are viewed perpendicularly to the helix axis, represented by *arrows*. The *bottom* representations are viewed along the axis. **a** The pitch value of 95 Å (*top*) corresponds to an angle of rotation about the axis and an axial separation between two subunits of, respectively, 58° (6.2 subunits per turn) and 15 Å. **b** The corresponding values are 83 Å for the pitch, 60° (6 subunits/turn) and 14 Å in the compressed form. The diameters of each filament form are indicated in the *bottom* views. The scheme in **a** only represents one possible organization of the subunits within the active filament (after Fig. 1 of Prévost and Takahashi 2003)

Beyond these similarities, several differences in nucleofilament activity have been observed, notably concerning the directionality of the reaction (Sung and Robberson 1995), the rate of ATP hydrolysis (Sung 1994; Li et al. 1997), or the conditions for ssDNA/dsDNA binding preference (Rice et al. 2001). It must be noted that the perception of recombinase activity in higher organisms is rapidly evolving with the exploration of optimal conditions for the Rad51 strand exchange reaction (Bugreev and Mazin 2004; Liu et al. 2004; Shim et al. 2006a,b) and with growing evidence for the active involvement of auxiliary proteins like BRCA2 (Galkin et al. 2005).

In this chapter, we will mainly refer to results observed with *Escherichia coli* RecA, since the collected data for this early discovered recombinase is much more important than for the other family members.

1.3

Protein/DNA Interactions Inside the Filament

There are at least two DNA binding sites inside the filament. The first site binds the ssDNA on which the subunits initially assemble, the second one interacts with the incoming dsDNA and binds the displaced strand after strand exchange, although not very strongly (Mazin and Kowalczykowski 1998; Gourves et al. 2001). The strand bound to the first site is only released upon filament dissociation. Details of the protein/DNA interactions in the two sites, and the nature of the amino acids participating in these interactions, have been systematically investigated for RecA using saturation mutagenesis, cross-linking experiments, and fluorescence studies (see Rehrauer and Kowalczykowski 1996; Takahashi et al. 1996; Prévost and Takahashi 2003 and references therein). Basically, two main zones inside the filament have been identified as binding DNA; they contain the flexible loops L1 and L2. Other zones of interaction are situated at the periphery of the filament and may correspond to entry points of the DNA into the filament. A recent study confirms the DNA binding role of loop L1 and the proximity of loop L2 to DNA in Rad51 filaments (Matsuo et al. 2006). In the first binding site, the single strand is mainly bound by its phosphodiester backbone, although the results of Volodin et al. (2003) on DNA phasing indicate that the nucleobases may also interact to some degree with the protein. Several results converge towards the participation of L1 to this site. Concerning loop L2, there are indications that this loop is situated close to the DNAs bound both in the first and in the second site, although it loses its proximity with the first DNA upon introduction of the second one (Wang and Adzuma 1996). These results are not surprising if one considers the space that L2 occupies within the filament interior. In the crystal structure of MvRadA (obtained in the absence of DNA), L2 comes close to the filament axis and when it is modeled as an extended β -hairpin⁶ it can easily “cross” the filament axis (Prévost and Takahashi 2003). Therefore, there is a strong probability that L2 may approach the ssDNA bound in the first site, which would then be clamped between L1 and L2 (Fig. 8 in Prévost and Takahashi 2003). In addition, it is hard to envision that the dsDNA can enter the filament without any interference from loop L2. Two situations can be envisioned: (i) the loop can find itself inserted between the ssDNA and the incoming dsDNA, in which case it may assist the strand exchange; or (ii) it

⁶ The isolated L2 peptide folds as a β -hairpin in the presence of DNA, to which it binds strongly as a filament (Wang et al. 1998; Selmane et al. 1999).

can withdraw during dsDNA introduction so that it guides the DNA without interfering with the dsDNA/ssDNA association. In both cases, it may interact with the displaced strand and assist its disconnection from the newly formed duplex.

1.4

Characteristics of Sequence Recognition in Homologous Recombination

Once a double strand break has been created, whether by accident or in order to initiate gene crossover, a genome-wide homology search process begins. The minimal search window of one end of a double-strand break has been estimated to be hundreds of nucleotides for *Drosophila* and mammalian cells, less than 100 nucleotides for bacteria and yeast (Engels et al. 1994 and references herein). One mismatch within a 100-nucleotide sequence window reduces mitotic gap-repair rates in *Drosophila* by a factor of seven (Nassif and Engels 1993). An influence of the base pair sequence on the search process is observed as early as the formation of the nucleofilament: some ssDNA sequences favor or hinder the binding of recombinases, and this influences the overall frequency of recognition. This particular aspect will be reviewed in Sects. 2 and 4.3.

More specifically, sequence recognition or discrimination is involved at several stages of the overall strand exchange reaction: during the encounter phase, during strand exchange, and upon stabilization of the newly formed duplex (the heteroduplex). Once strand exchange has been successfully initiated, recognition continues to be involved during the propagation phase, following a different mechanism assisted by ATP hydrolysis. Sects 3 to 5 report different models that have been developed to account for sequence effects during recognition. The scope of these models ranges from the nucleus to the atomic resolution, and from time scales of minutes to microseconds. The diversity of the reported points of view can be directly related to the sophistication of the mechanism of recognition and strand exchange, based on a subtle balance between thermodynamic stabilization and kinetic trapping. Such balance is required for the homologous search to be fast and to rapidly halt and resume when a zone of heterology is encountered.

In this chapter, we will concentrate on the characteristics of homologous recombination that are relevant to recognition. Many interesting reviews have covered other aspects of the HR reaction, and the reader can refer to them for further information concerning RecA (Howard-Flanders et al. 1984; Radding 1991; Kowalczykowski and Eggleston 1994; Takahashi and Nordén 1994; Roca and Cox 1997; Campbell and Davis 1999a; Prévost and Takahashi 2003; Bell 2005; Cox 2007a) and concerning other members of the RecA family and auxiliary proteins (Camerini-Otero and Hsieh 1995; Baumann et al. 1996; Kowalczykowski 2000; Kuzminov 2001; Cox 2007b).

2

Sequence Effects in Homologous Recombination

2.1

A Non-specific Reaction?

In a first approximation, the reaction of homologous recombination can be considered as non-specific, which means that it can accommodate any sequence of nucleobases. In contrast, the nucleofilament built on ssDNA is highly sequence-specific since it is able to recognize long stretches (up to kilobases) of homologous dsDNA sequence. These stretches are much longer than those recognized by any other sequence-specific DNA binding protein, where the size of the recognized sequence does not exceed 25 base pairs. However, these considerations need to be further nuanced. In fact, the reaction shows various dependencies on the ssDNA base pair content and sequence, and it tolerates a certain degree of heterology.

2.2

Sequence Effects in Recombinase–DNA Association

Even in the case of fully homologous sequences, some notable exceptions to the sequence universality of recombination have been observed. For example, it was shown that an increase of the GC content of DNA sequences from 16% to 40% halves the yield of recombination induced by RecA, while a 40% GC content practically inhibits the reaction induced by human Rad51 (Gupta et al. 1999b). This reduced rate was found to arise from an unfavorable strand exchange process, the rate of homologous pairing being unaltered with respect to more AT-rich sequences. Other works have specifically addressed the binding selectivity of DNA sequences by RecA (Tracy and Kowalczykowski 1996) and by Rad51 (Seitz and Kowalczykowski 2006) by sequence selection *in vitro*. It was found in both cases that the sequences preferentially recognized by the recombinase proteins are characterized by high G and poor AC contents. Moreover, the over-representation of G and T nucleotides in these preferred sequences increases with the complexity of the organisms (27% for bacteria, 32% for yeast, and 47% for human) (Seitz and Kowalczykowski 2006). In addition, when tested for strand exchange, the G,T-rich sequences showed enhanced rates as compared with sequences poorly recognized by recombination proteins. A notable exception is encountered for dinucleotide repetitions poly-(GT), also called microsatellites (Duttreix 1997). Microsatellites still bind recombinases with high affinity, but they have been found to slow or even inhibit RecA-induced homologous recombination *in vitro*, and to inhibit meiotic recombination *in vivo* in yeast *Saccharomyces cerevisiae* (Gendrel et al. 2000). An interpretation of this effect of sequence repetition has recently been proposed, based on theoretical simulations of the recog-

nitiation process (Fulconis et al. 2005, see Sect. 4.1). The work from Volodin and collaborators brings more precision about the interaction of recombinases with G and T bases (Volodin and Camerini-Otero 2002; Volodin et al. 2003). The authors showed that G,T-rich DNA sequences as short as eight nucleotides influence the phasing of RecA subunits on DNA, i.e., each RecA filament in solution is positioned in the same way with respect to the DNA sequence. This indicates some degree of specificity in the RecA/G,T interactions. The phasing is destroyed by the replacement of G,T by A,C nucleotides.

2.3

Tolerance for Heterology in RecA-Catalyzed DNA Recognition and Strand Exchange

A certain degree of heterology can be tolerated both in the phase of initiation and in the phase of propagation of RecA-mediated strand exchange; however, with different characteristics. During the phase of initiation, isolated heterologies are tolerated in up to 10% of the sequence, although they decrease the rate of strand exchange (Bucka and Stasiak 2001). For example, the presence of 12 non-consecutive substitutions distributed in a 83-mer oligonucleotide results in a sixfold decrease of the reaction rate (Bazemore et al. 1997a). In the second phase, after strand exchange has been successfully initiated on a homologous stretch of the incoming dsDNA, insertions and deletions in the dsDNA can be bypassed during strand exchange propagation with the help of ATP hydrolysis (Bianchi and Radding 1983; Rosselli and Stasiak 1991). Bucka and Stasiak (2001) have proposed an interesting interpretation of this differential tolerance. According to the authors, tolerance to isolated mismatches during homologous recombination is made necessary by the occurrence of stochastic base mutations in protein-coding genes. Base substitutions are much less harmful to the genome than base insertions or deletions since they do not modify the phasing of the codons⁷. Besides, simple nucleotide substitution can also express the degeneracy of the genetic code. In such cases, the homologous register of the aligned dsDNA and ssDNA is conserved during homologous recombination.

Both the nature and the position of substituted bases have an effect on the completion of the strand exchange reaction. Malkov and Camerini-Otero (1998) have investigated the kinetics of dissociation of RecA protein/three-stranded DNA complexes where the ssDNA presented isolated heterologies. They have classified the different types of substitutions introduced between the ssDNA and the dsDNA sequences according to their effect on the rate of dissociation. The ranking order was found different whether the substitutions had been introduced at the 5'-end or at the 3'-end of the oligonucleotide. Recent work confirm the importance of the substituted bases being located at the 5'- or 3'-end (Sagi et al. 2006).

⁷ In vivo, anti-recombination proteins like MutS and MutL help to avoid frameshifts.

Taken together, the available information on sequence recognition and discrimination does not allow one to rationalize the sequence effects on strand exchange or to extract predictive rules for recognition. The following sections present different models that have been developed in order to assist the interpretation of these data.

3

Homology Search in the Cell

The search for a sequence homologous to that of a given nucleofilament, within the whole genome of *Escherichia coli*, is successfully performed in less than 1 min in vivo. This is a very short time if one considers the size of this genome. Several scenarios are possible to account for homology search at the cell level, given that the nucleofilament must find its target sequence among a large number of non-specific sequences (Dutreix et al. 2003). Since it is known that the nucleofilament interacts weakly with non-homologous sequences, a “sliding” hypothesis has first been proposed. This implies that the nucleofilament binds non-specifically to the DNA and then diffuses linearly along its whole length until it finds the correct sequence. This scenario has been tested and rejected by Adzuma (1998). As an alternative proposition, the search may be assisted by the formation and the stabilization of coaggregates (Fig. 3b) between DNA stretches and the nucleofilament (Levin-Zaidman et al. 2000; Dutreix et al. 2003), thus limiting the time spent on diffusion. The groups of Dutreix and Viovy (Dutreix et al. 2003; Dorfman et al. 2004) have proposed an analytical recognition model that captures the essential features of the search for homology in HR. Because of the huge size of both partners and the rigidity of the nucleoprotein filament with respect to the DNA, the authors propose that the relevant searching species is the DNA, more specifically a DNA segment of the “Kuhn length”⁸, which randomly contacts the nucleofilament (Fig. 3c). The model incorporates the following aspects of homology search: (i) recognition can initially take place on short segments, situated anywhere in long homologous regions, and more than one segment can be recognized at the same time; (ii) the corresponding bases between the duplex DNA and the nucleofilament do not initially share the same phasing, due to the stretching by 50% of the bound ssDNA; (iii) the dsDNA must be at least locally stretched for the recognition to take place; and (iv) local sliding can take place during the time interval where a Kuhn fragment is close to the nucleofilament. Starting from these characteristics and using a robust physical model for the diffusion of long polymers in a crowded en-

⁸ The “Kuhn length” of a polymer chain is defined as twice its persistence length. The persistence length quantifies the stiffness of the polymer submitted to Brownian forces, it is defined as the length over which the tangent direction remains correlated. The persistence length of duplex DNA is 50 nm. For RecA/ssDNA filaments, it has been estimated to be 920 nm (Hegner et al. 1999).

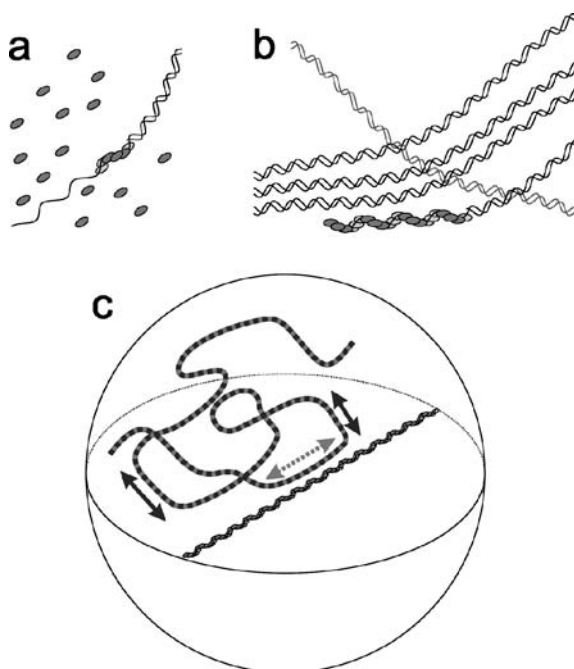


Fig. 3 Schematic steps of recognition in HR. **a** Recombinases polymerize on a single-stranded DNA in the presence of ATP, giving way to **b** a nucleoprotein filament where the ssDNA is stretched by a factor 1.5. Networks of dsDNA/nucleofilament can form by co-aggregation. **c** Kuhn fragments of dsDNA (indicated by a *broken arrow*) randomly contact the filament and stay aligned with it during a short period of time, due to non-homologous interactions (adapted from Dutreix et al. (2003), Dorfman et al. (2004))

vironment, together with estimated values for the energy of non-specific and specific nucleofilament/DNA interactions, the model predicts a search time that is compatible with biological data⁹. The results strongly suggest a length of three nucleotides for the initial recognition fragment. The model also permits evaluation of the effects of environmental parameters like the viscosity or the strength of the non-specific interactions, which depend on the ionic concentration. Finally, it predicts a first-order kinetics in the case of long DNA, with a linear increase of the kinetic rate with the size of the DNA, which agrees with experimental results on the global kinetics of recognition (Julin et al. 1986; Lankenau et al. 2000). Note that Patel and Edwards (2004) reach the same kinetic result using a numerical approach applied to a flexible DNA and a nucleofilament considered as linear.

⁹ The search time has been experimentally evaluated to be less than 1 min for a 6.4 kb single strand to perform a search on a duplex DNA of the same size (Honigberg et al. 1986). The approximate search time obtained by the analytical calculations of Dorfman et al. (2004) is about 500 s in the hypothesis of a three-base pair recognition seed.

Klapstein and Bruinsma (Klapstein et al. 2004) have investigated how the stiffness of the recombination filament and the stretching of DNA influence the kinetics of the search for homology. Based on topological considerations (lack of register of the ssDNA and dsDNA to be recognized, geometrical conditions for multiple points of simultaneous recognition) and on the known stiffness characteristics of RecA-DNA filaments (Hegner et al. 1999), their physical calculations indicate that the two components are determinant in driving the recognition and exchange processes to completion in an efficient and rapid way. The fact that the RecA-bound ssDNA is stretched increases the probability for an ssDNA fragment and its homologous dsDNA fragment to fall in register. Moreover, the calculations show that DNA stretching, combined with the RecA filament stiffness, precludes the apparition of simultaneous seeding points for recognition at several places of the sequence.

4

Models of Homology Search at the Molecular Level

Once the searching DNA portion is positioned close to the nucleofilament, in such a way that it can transiently be captured by non-specific attracting forces, a new phase of homology search begins at the local level. What is at stake here is the ability to align and recognize homologous sequences, but also the capacity to discriminate misalignments and punctual sequence heterologies. Issues regarding the initiation and the propagation of homology recognition, as well as the bypass of heterologies, can be addressed at a molecular level independently of the characterization of the precise interactions between protein and DNA functional groups (discussed in Sect. 5).

4.1

Dynamic Monte Carlo Approach: A Numerical Model of Recognition at the Molecular Level

The recognition process that takes place during the initiation phase does not require any energy transduction. Nevertheless, it constitutes the limiting step of homologous recombination. Possible scenarii of recognition at the molecular level have been explored by the group of Viovy (Fulconis et al. 2005). In their approach¹⁰, which follows their analytical recognition model presented in Sect. 3 (Dorfman et al. 2004), energy terms for homologous or heterologous interactions enter as parameters of the model, together with terms linked to dsDNA-induced deformation (local stretching, stretching cooperativity). DNA deformation is taken into account by submitting nucleotides

¹⁰ Monte Carlo methods are commonly used for the numerical simulation of mathematical or physical processes described by many variables, which cannot easily be solved analytically.

to local compression or extension corresponding to thermal fluctuations (Fig. 4a)¹¹. Each base pair is free to establish or to break an interaction with the closest base of the stretched RecA-bound ssDNA and the model allows taking into account the effect of DNA sequence. This numerical treatment appears sufficiently robust to capture the basic features of recognition. The typical simulation results indicate a slow nucleation phase involving a few nucleotides, followed by rapid propagation to the rest of the sequence (Fig. 4b). The model is sensitive to the presence of heterologies, which increase the time of nucleation. Moreover, it provides an interpretation for the low rate of meiotic recombination observed with repeated sequences. Such sequences would trap the search in partially homologous configurations, characterized by out-of-phase misalignment (Fig. 4b).

4.2

Role of ATP Hydrolysis in Recognition and Strand Exchange

In the case of RecA-induced HR, ATP hydrolysis is involved only in the propagation phase of the reaction (Rosselli and Stasiak 1990), after initiation has successfully taken place (Kowalczykowski and Krupp 1995). It allows strand exchange to propagate along filaments comprising several kilobases, to bypass heterologies (Kim et al. 1992a) or to perform four-stranded reactions (Kim et al. 1992b). Topological difficulties arise after a certain length of DNA strands has been exchanged as a consequence of various events, like nucleation simultaneously taking place at different locations along the filament, insertion stretches needing to be looped out of the filament or, in the case of four-stranded reactions, a difference in helicity between exchanging and non-exchanging DNA strands. In order to release the resulting stress, the recombination filament acts as a molecular motor fueled by ATP hydrolysis, whose mode of functioning has been the subject of various studies. Radding (1991) has proposed a system of concerted rotations in opposite directions for the two exchanging DNA species within the filament. The group of Kowalczykowski hypothesized a mechanism of local redistribution, where the RecA subunits would dissociate in their RecA/ADP form and re-associate as RecA/ATP (Kowalczykowski and Krupp 1995). Such polymerization/depolymerization mechanism could, however, not be observed experimentally. The Cox group favors a “facilitated rotation model”, where the rotation of DNA takes place outside the protein filament (Cox 1994, 2003). This DNA may be the fourth strand of a four-stranded reaction, or it may be a dsDNA section in the case of multiple nucleation points. Cox proposes

¹¹ Léger et al. (1998) have observed that RecA polymerization on a dsDNA is facilitated when the oligonucleotide is previously stretched under micromechanical restraints to reach the S-DNA form (for “stretched DNA”; this form results from a structural transition observed under external tension (Cluzel et al. 1996)). Their calculations suggest that spontaneous thermal stretching fluctuations of the DNA may play a role in the binding of RecA to DNA.

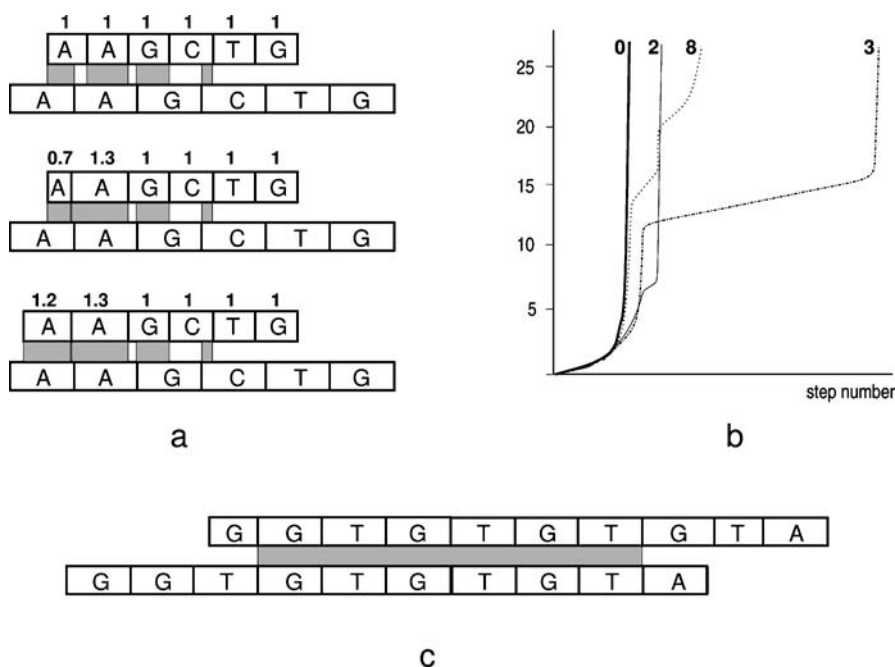


Fig. 4 Model for the search for homology within RecA filaments (Fulconis et al. 2005). At each position in the sequence, the local interbase extension varies by a factor ranging between 0.7 and 1.9 with respect to its size in B-DNA (3.4 Å). The extension of S-DNA (see Footnote 11) is 1.7 and the extension in RecA/bound ssDNA is 1.5. **a** From *top* to *bottom*, schematic representation of two consecutive Monte Carlo (MC) search steps. The ssDNA sequence (uniform extension 1.5) is represented below the searching ssDNA. Shaded areas between the bases represent the local degree of sequence homology. An MC step consists of modifying the relative extension of two consecutive base pair steps situated inside the DNA (*top* to *middle*) or the extension of one terminal base pair step (*middle* to *bottom*). **b** Typical behavior of the number of correctly paired bases (*vertical axis*) versus the number of MC steps (*horizontal axis*) in typical MC simulations performed on short oligonucleotides (~25 nucleotides) using fully homologous sequences (*bold line*), two consecutive (*thin line*), three consecutive (*broken line*) or eight non-consecutive (*dotted line*) mismatches. **c** Example of partial recognition in the case of repeated dinucleotide sequences, which can trap the search for homology

that it interacts with binding sites outside the filament and that it is rotated to a neighboring site each time a cofactor is hydrolyzed. This model has provided the first interpretation for the high rate of ATP hydrolysis by RecA nucleofilaments.

More recently, the group of Bruinsma (Klapstein and Bruinsma 2000) proposed a model based on the fact that two non-interconvertible forms for the RecA filament have been identified, depending on whether the cofactor is ATP ("extended" form) or ADP ("compressed" form), respectively (Yu and Egelman 1992). These forms are characterized by different subunit/subunit

interfaces (VanLoock et al. 2003). Klapstein and Bruinsma (2000) propose that ATP hydrolysis generates an elastic stress along the filament, due to the fact that it is impossible for RecA/ADP to adopt the equilibrium form it occupies in the compressed form. This stress would be capable of inducing a force on DNA at the point of strand exchange. As for the Cox model, the strand exchange reaction would proceed via waves of concerted monomer hydrolysis, acting as rotary motors and propagating along the length of the filament.

4.3

The Kinetics of Homology Search

Beyond their remarkable capacity to capture the dynamic dimension of the search progression during initiation and propagation, the models presented in Sects 4.1 and 4.2 consider the search for homology as a global event. They do not discriminate between the different phases of the reaction, i.e incorporation of the dsDNA in the nucleofilament and strand exchange. Several kinetic studies performed during the last 10 years have been able to monitor the reaction along these steps (Bazemore et al. 1997b; Gumbs and Shaner 1998; Xiao et al. 2006). The purpose was to detect at which point of the reaction the substituted bases are discriminated. Up to three reaction intermediates have been identified, using combined stop-flow spectrofluorometry and fluorescence resonance energy transfer (FRET) experiments, each time more sophisticated¹². It was found that the insertion of substitutional heterologies in the dsDNA substrate influences each reaction step, however, in different manners. While the rate of formation of all three intermediates appeared significantly reduced, particularly in the case of consecutive mismatches, the dissociation rate of the early, very short-lived reaction intermediate was found to be independent of the number of substitutions (Lee et al. 2006). On the contrary, for the second intermediate, the decrease of the equilibrium constant with the number of mismatches appeared to result from a combination of both a decreased rate of formation and an increased rate of dissociation. From this data, Bazemore et al. (1997a) proposed an interpretation for the recognition process involving two stages, decomposed into a moderately discriminative dsDNA/ssDNA association stage (first two kinetic steps) and a strand exchange stage (third step) to complete the recognition.

Not only the number of mismatches but also the nature of the exchanged base pairs influences the kinetics of RecA-induced strand exchange. The group of Radding was able to specifically measure the rate of exchange of A,T

¹² These intermediates are relevant to the initiation step and do not depend on whether the cofactor can be hydrolyzed or not. Gumbs and Shaner (1998) have shown that the kinetics of the reaction performed with ATP or its almost non-hydrolyzable analog ATP γ S as a cofactor only varies for the last step, where the displaced strand is released. When the cofactor is ATP γ S, this strand stays inside the filament.

bases by replacing each adenine of an oligonucleotide by a fluorescent analog, 2-aminopurine (Folta-Stogniew et al. 2004). Interestingly, it was observed that A:T base pair disruption in the substrate dsDNA occurs simultaneously to the formation of new Watson–Crick interactions in the product heteroduplex. Identical observations were obtained with Rad51 (Gupta et al. 1999a). According to Voloshin and Camerini-Otero (2004), the recognition process would take advantage of the spontaneous and transient opening events, or breathing, which are favored in A:T base pairs.

Taken together, the results of this ensemble of kinetic studies picture a complex mechanism, where partial recognition coincides with local internal rearrangements of the intertwined three strands, and where A:T and G:C base pairs play different roles, exchanging Watson–Crick pairing in a one-step process (A:T) or giving way to a two-step recognition process (G:C) (Voloshin and Camerini-Otero 2004).

5 Homology Recognition at the Atomic Level

5.1 Hypothesis

Understanding recognition promoted by recombinase nucleofilaments requires the identification of the exact interactions occurring between the DNA strands, and accessorially between the DNA strands and the protein monomers. The ssDNA strand is known to be bound within the filament by its phosphodiester backbone, with its bases exposed to the solvent and free to interact with the incoming dsDNA (Nishinaka et al. 1997). Recognition then involves direct interactions between functional groups of the two DNA species. Two extreme scenarios have been proposed (Howard-Flanders et al. 1984). The dsDNA chains can separate before ssDNA anneals with its complementary strand, in which case recognition simply results from Watson–Crick reading between the bases of the ssDNA and the complementary strand. This hypothesis is supported by the fact that DNA stretching favors base pair opening, although opening has never been observed in the absence of strand exchange. Alternatively, recognition can directly occur between the ssDNA and the intact dsDNA, in which case the ssDNA bases necessarily sense the dsDNA sequence in one of the grooves. This implies the transient formation of a triple helix as the recognition species¹³ (Howard-Flanders et al. 1984; see also Camerini-Otero and Hsieh 1993; Rao and Radding 1993). Whether the ssDNA would

¹³ Recombination triple helices differ from classical triple helices by the fact that the two strands with identical sequences are parallel in the first case and antiparallel in the second case. Discovered in the 1950s, classical triple helices are used in anti-gene strategies (Garestier et al. 1996).

be positioned in the minor groove or in the major groove of such a putative triple helix has long been a subject of controversy.

In the light of the kinetic results of the Radding group (Folta-Stogniew et al. 2004) and more recently of Lee et al. (2006) (Sect. 4.3), the real mechanism is likely to lie between these two extreme scenarios, more precisely to be composed of a mixture of the two. While the results of Lee et al. (2006) strongly argue against the formation of long triple helices as intermediates of recognition and confirm that recognition involves Watson–Crick reading of melted base pairs in the case of A,T stretches, they still favor an initial recognition of intact G:C base pairs by the ssDNA bases. The question of which groove is approached therefore remains of importance. It is also critical to gain information on the nature and properties of G,C-containing base triplet arrangements that may locally form in early recognition intermediates. In this perspective, previous investigations bearing on the formation and the properties of putative parallel forms of triple helices of recognition can bring useful insights in understanding the recognition mechanism.

5.2

Looking for Reaction Intermediates

Major groove parallel triple helices called R-DNA (where R stands for recombination) (Zhurkin et al. 1994) have been isolated and characterized in certain reaction conditions, specifically when the 5'-extremity of the displaced strand was impeded from leaving the filament¹⁴ and provided that the RecA cofactor was hydrolyzed (Jain et al. 1995). These triple helices have initially appeared as attractive recognition intermediates, where each base of the ssDNA would unambiguously interact with its homologous base pair via strong interactions in the major groove (Zhurkin et al. 1994). Nevertheless, these isolated products may also correspond to the result of strand exchange. Indeed, experimental evidence accumulates in favor of a minor groove approach of the dsDNA (Baliga et al. 1995; Podyminogin et al. 1996; Zhou and Adzuma 1997; Xiao and Singleton 2002). The most direct indication comes from the structural characterization of a post-exchange reaction intermediate (Xiao and Singleton 2002), based on fluorescence energy transfer experiments. In this intermediate, the displaced strand is positioned in the major groove side of the product heteroduplex.

We have previously shown that the theoretical construction of a minor groove triple helix of recombination is possible, although such a helix can only exist in a stretched form, the ST-DNA helix (where ST stands for “stretched triple”) (Bertucat et al. 1998, 1999). When stretching a duplex DNA, it becomes possible to incorporate a single strand in its widened mi-

¹⁴ Depending on the experiment, the displaced strand was either covalently linked to its former complementary strand or it followed a zone of heterology.

nor groove. This in turn locks the helix in the stretched ST-DNA form. While calculations indicate that the ST-DNA triple helix is metastable, it can be stabilized via the exchange of pairing interactions within each base triplet, facilitated by the relative position of the bases (Figs. 5a and 5b, top). For the exchange to occur, only a limited (50°) in plane rotation of the complementary base (i.e., the base which switches its partners) around its phosphodiester backbone is necessary (see Fig. 5b). Numerical calculations indicate that this process is exothermic. Finally, simulating the pairing exchange of all base triplets in a ST-DNA helix leads to the R-DNA triple helix described above.

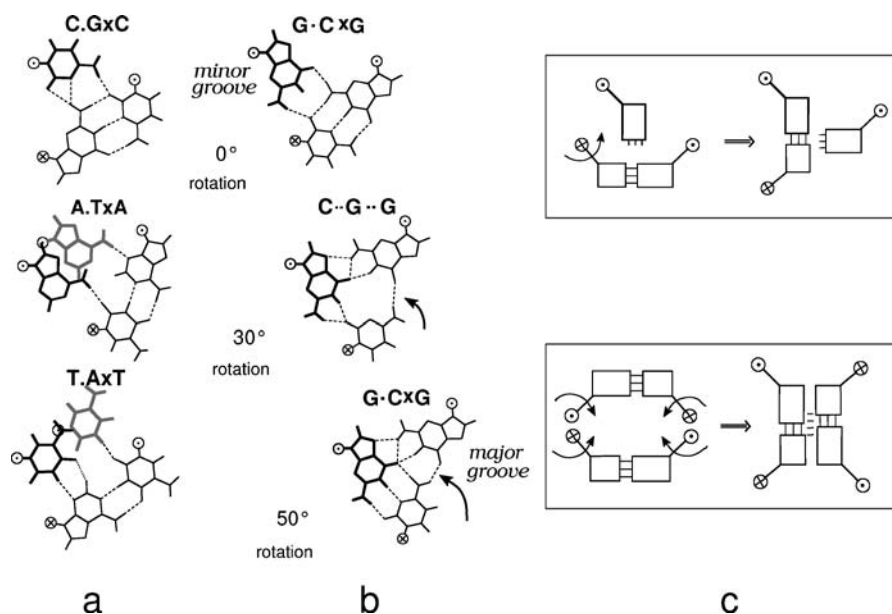


Fig. 5 Model of recognition and strand exchange via a minor groove triple helix putative intermediate, ST-DNA. **a** Patterns of minor groove interaction for C.GxC, A.TxA and T.AxT base triplets. In the last two cases, an alternative position found for the base in the minor groove is represented in gray. Hydrogen bonds are represented by dotted lines. DNA backbones pointing upwards or downwards are indicated by dots and crosses, respectively. **b** Pairing exchange within a G.CxG base triplet, modeled by rotating the cytosine around its phosphodiester backbone. The guanine which initially belongs to the ssDNA, is in bold. From top to bottom are represented three stages of pairing exchange. In the initial stage, the ssDNA base is in the minor groove of the dsDNA; in the middle stage (30° rotation) the three bases interact within a cyclic intermediate; in the final stage (50° rotation) the cytosine has switched its Watson-Crick partner and the displaced guanine is in the major groove of the newly formed base pair. **c** Schematic models of strand exchange for a three-stranded reaction (top) and for a four-stranded reaction (bottom). In this model, a concerted rotation of the four bases results in the formation of new Watson-Crick bonds between the two initial duplexes, together with the creation of side-by-side interactions in the two major grooves

Calculations also show that sequence discrimination in the minor groove of ST-DNA triplexes is limited to G,C-containing base pairs, while discrimination of heterologies involving A,T base pairs rather occurs during pairing exchange or within the product heteroduplex (Bertucat et al. 2000). Depending on the flanking sequences, pairing exchange of A and T bases could be observed at little or at no activation energy cost, indicating that interaction and exchange can occur simultaneously, while the activation energy was much higher for G and C bases (Bertucat and Prévost, unpublished results). It is interesting that these calculation results meet the kinetic observations of Folta-Stogniew et al. (2004) and Lee et al. (2006). Finally, the local formation of a minor groove triple helix is further supported by the similarities between the stretched and unwound duplex part of this helix and the structure of DNA stretches contacting minor groove binding proteins (Prévost and Takahashi 2003).

When exploring possible topologies of the DNA strands, it is interesting to refer to the purely theoretical, early model for strand exchange proposed by McGavin (1971) and further developed by Wilson for the construction of stable quadruplexes starting from two duplexes (Wilson 1979) (Fig. 5c, bottom). Interestingly, although there was no structural information available at that moment from experimental studies, Wilson found that in order to avoid steric hindrance, the duplexes had to be stretched and unwound before they were aligned by their minor groove side. Exchange of the homologous strands would then allow the quadruplex to rewind and recover a B-like geometry¹⁵. While there is presently no evidence for the presence of transient quadruplexes inside recombination filaments, the topological considerations remain pertinent when applied to the three incorporated DNA strands.

Very recently, Richard Egel proposed a model that reconciles the minor groove and the major groove hypotheses described above by exploring the possibility of strand exchange occurring via transitory base pairing in a *syn-syn* configuration (Egel 2007). The *anti-syn* base transition would be made possible by the high degree of DNA stretching. The model relies on a quasi-quadruplex symmetry for the exchange of two homologous duplexes, with the fourth strand lying outside the RecA filament scaffold.

It remains to be experimentally established which elements of these possible scenarios enter in the real recognition processes and how they combine with the dynamics of strand exchange revealed by the kinetic studies. A direct participation of the protein itself, beyond inducing the initial stretched and unwound DNA conformation, must also be considered. Particularly, the possible role of the two flexible loops contacting the DNA (Sect. 1.3), and close to the ATP binding site for the longest one, must be thoroughly examined, together with the overall conformational changes in the filament associated with ATP hydrolysis (Sect. 4.2). Here also, the dynamics of strand exchange

¹⁵ Such a quadruplex has since been modeled by Lebrun and Lavery (1995).

make these elements difficult to directly assess experimentally and it is hoped that model building will bring useful insights.

6

Conclusion

The search for homology by recombinase filaments requires both high velocity, for scanning the whole genome in a reasonable time interval, and a high level of accuracy for sequence homology recognition. In order to apprehend the subtle process capable of achieving both these goals, it is necessary to simultaneously take into account different levels of resolution (cellular, molecular and atomic) and different aspects of the process (static and dynamic). It is now established that recognition cannot be studied independently of strand exchange, and so the quest for characterization of strand exchange intermediates continues to be a major challenge. In the light of new data indicating different kinetics for A:T and G:C base pairs, this challenge turns out to be even more complicated than expected when considering the rapidity of the reaction. In this respect, model building and numerical simulation of the physical process appear to be promising tools. The field has altogether testified impressive progress in recent years. One can anticipate that refined multiscale approaches integrating experimental knowledge will finally permit the capture and interpretation of the recognition and strand exchange mechanisms, finally leading to predictive applications.

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Biochemistry of Meiotic Recombination: Formation, Processing, and Resolution of Recombination Intermediates

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Abstract Meiotic recombination ensures accurate chromosome segregation during the first meiotic division and provides a mechanism to increase genetic heterogeneity among the meiotic products. Unlike homologous recombination in somatic (vegetative) cells, where sister chromatid interactions prevail and crossover formation is avoided, meiotic recombination is targeted to involve homologs, resulting in crossovers to connect the homologs before anaphase of the first meiotic division. The mechanisms responsible for homolog choice and crossover control are poorly understood, but likely involve meiosis-specific recombination proteins, as well as meiosis-specific chromosome organization and architecture. Much progress has been made to identify and biochemically characterize many of the proteins acting during meiotic recombination. This review will focus on the proteins that generate and process heteroduplex DNA, as well as those that process DNA junctions during meiotic recombination, with particular attention to how recombination activities promote crossover resolution between homologs.

Abbreviations

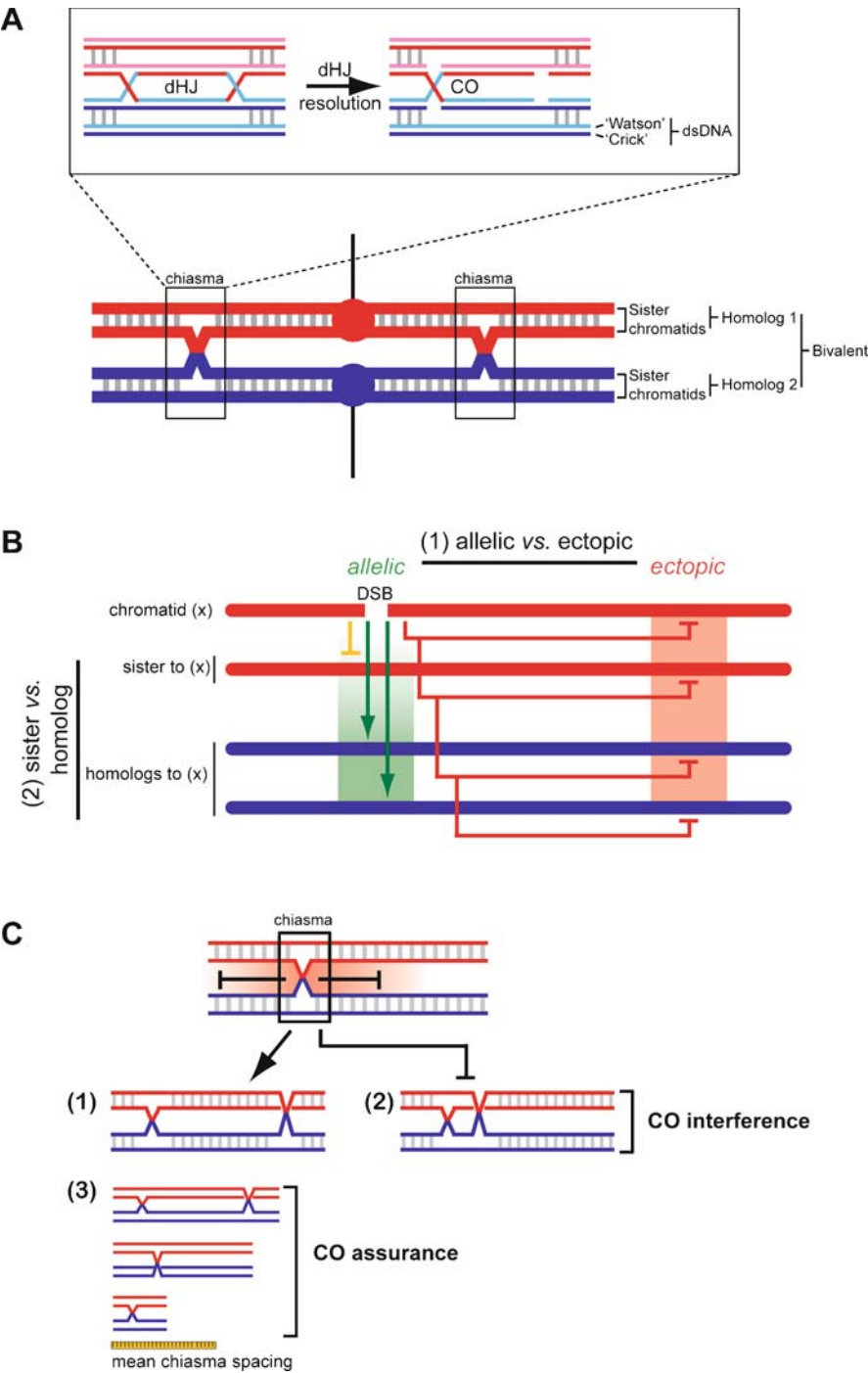
ChIP	chromatin immunoprecipitation
CO	crossover
dHJ	double Holliday junction
DSB	double-stranded DNA break
DSBR	double-stranded DNA break repair model
dsDNA	double-stranded DNA
hDNA	heteroduplex DNA
HJ	holliday junction
HR	homologous recombination
MMR	mismatch repair
MRX	Mre11-Rad50-Xrs2
MRN	MRE11-RAD50-NBS1
MRX/N	Mre11-Rad50-Xrs2 or Mre11-Rad50-NBS1
NCO	non-crossover
NHEJ	nonhomologous end-joining
SDSA	synthesis-dependent strand annealing model
SEI	single-end invasion
SSA	single-strand annealing
ssDNA	single-stranded DNA

1

Introduction

HR plays a critical role during meiosis to ensure that the paternal and maternal homologs segregate from one another during the first meiotic division. This is achieved by the physical connections between homologs, called chiasmata, that are formed as a consequence of crossovers (COs) generated during meiotic recombination (Fig. 1, part A). Genetic analysis of tetrads and octads resulting from fungal meiosis led to the Holliday model (Holliday 1964) and its revisions to the present version shown in Fig. 2, the DSBR-SDSA model (see chapter by J.E. Haber, this BOOK, on the evolution of recombination models). The central tenets of the Holliday model, heteroduplex DNA and four-way cross-stranded junctions, or Holliday junctions (HJs), still represent the critical intermediates during meiotic recombination. Here, we focus on the biochemistry of meiotic recombination as it pertains to the formation

Fig. 1 Meiotic crossovers establish physical connection between homologs. **A** The two homologs each consist of two sister chromatids (*red and blue lines* each depicting a dsDNA molecule) held together by cohesion (represented by *grey lines* between the sisters). Upon bipolar attachment of the kinetochores (*red/blue circles*) to the meiosis I spindle (*black lines*), the CO points between homologs (indicated as chiasmata) provide a counterforce to the spindle force acting on the kinetochores, signaling correct bipolar attachment of the paired homologs (bivalent) and ensuring high-fidelity chromosome segregation during meiosis I division. Resolution of a double-Holliday junction (dHJ) by alternate incision, as shown in the *box*, represents a mechanism to generate a chiasma. The individual DNA strands involved are shown in the *box*. **B** Meiotic recombination entails objectives unique from those in vegetative cells. (1) As in vegetative cells, recombination is minimized between ectopic sequences but is instead directed toward allelic sites. Mechanisms responsible for the biochemical differentiation between ectopic (homeologous) and allelic (homologous) sites are poorly known but probably involve mismatch repair factors and regulation at the level of heteroduplex quality during preliminary DNA strand exchange events. (2) Meiotic recombination promotes a regulated level of DSB repair directed to the homolog, at the exclusion of the sister chromatid. The biochemical basis of sister vs. homolog discrimination is also poorly understood and remains an outstanding question for recombination applications specific to meiosis. **C** Crossovers are an essential outcome of the meiotic recombination agenda, but only under strict limitations of number (incidence) and distribution. Where one CO occurs in a bivalent, the probability of a second CO nearby is far below what would be expected by random distribution. This suggests that the number and spacing of COs is regulated; a phenomenon known as CO (or chiasma) interference. It is unclear at what level (pre-DSB, DSB, SEI, dHJ) interference is imposed. Not all organisms display interference (e.g., *Schizosaccharomyces pombe* does not) (Munz 1994), and not all CO pathways are associated with interference (see Fig. 12). Although the underlying mechanism(s) for CO interference remain to be explained, interference results in the non-random spacing of chiasmata on chromosomes that undertake multiple CO events (1) and (2). Interference may also play a role in CO assurance (3), the observation that all bivalents earn at least one chiasma, even on chromosomes that are smaller than the mean chiasma spacing



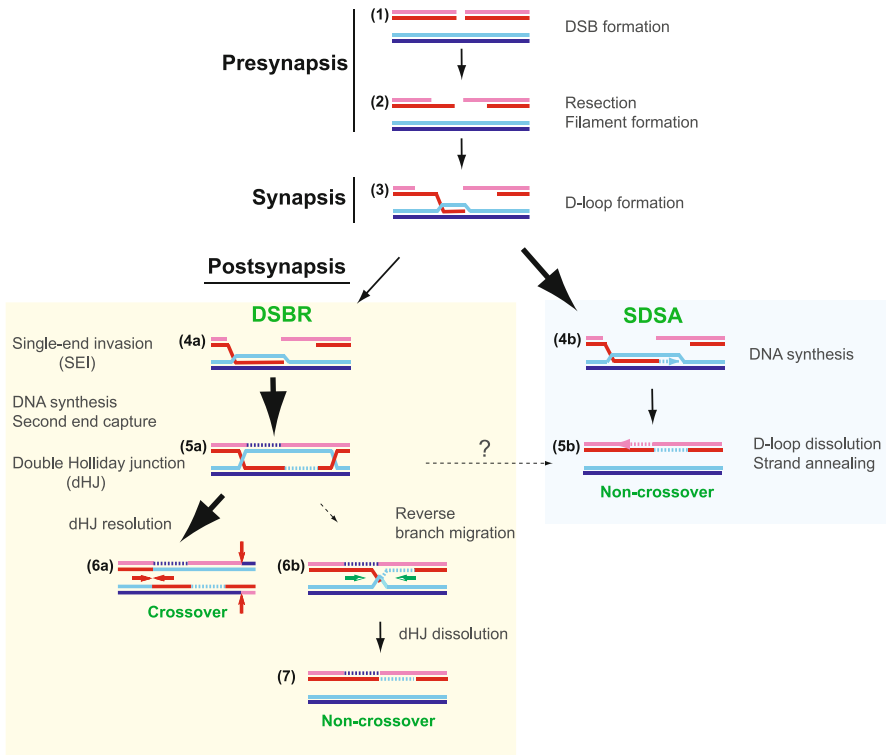


Fig. 2 Mechanistic stages of homologous recombination. Meiotic recombination is initiated by a Spo11-mediated double-stranded DNA break (DSB) (1). During presynapsis, the initial break is resected to form 3'-OH ending single-stranded DNA tails to allow formation of filaments by the DNA strand exchange proteins, Rad51 and Dmc1 (2). During synapsis, a joint molecule is formed between the broken DNA and an unbroken template from the other homolog, positioning the 3'-OH end for DNA synthesis (3). During postsynapsis, meiotic recombination bifurcates into at least two primary pathways that repair the DSBs: most breaks are repaired to NCO products by SDSA, but a fraction of breaks are repaired to CO products by DSBR. SDSA (4b, 5b) dissolves the initial D-loop to reanneal the extended invading strand to the second end of the break site, resulting in NCO products (Nassif et al. 1994; Resnick 1976). Second end capture and dHJ formation (DSBR, 5a, 6a,b, 7) (Szostak et al. 1983) account for the main CO pathway in budding yeast, nematodes, and mammals (termed CO pathway 1). Possible scenarios for CO pathway 2 (predominant in fission yeast) and 3 (predominant in *Drosophila*) are shown in Fig. 12. The joint molecule physically identified as the SEI intermediate (4a) appears to be a stabilized D-loop and is a CO-specific intermediate in meiosis (Hunter and Kleckner 2001). The dHJ intermediate (5a) is critical for CO formation, possibly through resolution by structure-specific endonucleases resembling the bacterial RuvC enzyme (6a). Resolution of dHJs might be biased to CO products, such that there is no NCO outcome ("?" in 5a to 5b transition). Alternatively, a minor fraction of dHJs may be dissolved into NCO products by a RecQ-family helicase, a topoisomerase III, and a junction specificity factor, involving reverse-branch migration that confines heteroduplex DNA to the recipient chromosome (6b–7) (Wu and Hickson 2003) (see Fig. 11)

and processing of the heteroduplex DNA and DNA junction intermediates (see Table 1 for a list of proteins that are discussed). Additional important aspects of meiotic DNA transactions are discussed in more depth in other chapters, including the initiation of meiotic recombination by DSB formation (S. Keeney, this SERIES) and the mechanism of homology search by RecA-like proteins (C. Prévost, this BOOK). This review will concentrate on results with the budding yeast *Saccharomyces cerevisiae* (Table 1), with references to other organisms where the proteins or mechanisms appear to differ from budding yeast. The chapters specifically dedicated to organisms, including fission yeast *Schizosaccharomyces pombe* (G. Cromie and G.R. Smith, this BOOK) and *Arabidopsis thaliana* (G.H. Jones and F.C.H. Franklin, this SERIES), will offer more detail on these systems. The reader is also referred to excellent previous reviews on meiotic recombination (Cromie and Smith 2007; Gerton and Hawley 2005; Hunter 2007; Krogh and Symington 2004; Orr-Weaver and Szostak 1985; Paques and Haber 1999; Roeder 1997; Zickler and Kleckner 1999).

2

Biochemistry of Meiotic Recombination

The *RAD52* epistasis group (*RAD50*, *RAD51*, *RAD52*, *RAD54*, *RDH54/TID1*, *RAD55*, *RAD57*, *RAD59*, *MRE11*, *XRS2*) forms the core of the recombination pathway in somatic and meiotic cells, aided by context-specific factors (Table 1). Meiotic recombination differs from recombination in somatic (vegetative) cells in significant aspects. First, recombination is strongly induced in meiosis (100- to 10000-fold), as we now know by DSBs introduced by the Spo11 protein (Figs. 1, part B, 2, 3). A similar increase in recombination (up to 4000-fold) in vegetative cells is induced by DSBs during gene targeting in budding yeast (Orr-Weaver et al. 1981). Second, meiotic recombination is designed to favor homologs over sisters, which are the preferred template for DSB repair in somatic cells (Fig. 1, part B). A third unique aspect of meiotic recombination in most eukaryotes concerns meiotic CO control and interference (Fig. 1, part C). Interference, precisely chiasma interference, defines the observation that a CO affects the probability of a second CO in its vicinity. The earliest studies on meiotic recombination in *Drosophila* established the existence of positive interference, showing that exchange (CO) in one interval decreased the probability of exchange (CO) in a nearby interval (Muller 1916; Sturtevant 1915). The mechanistic bases for the homolog bias and CO outcome of meiotic recombination are possibly related, as they both serve to establish the physical connection between homologs in the bivalent (Fig. 1, part A) that ensure proper chromosome segregation during the first meiotic division. These mechanisms likely involve meiosis-specific chromosome structures including the synaptonemal complex (Zickler and Kleckner 1999), meiosis-specific proteins, including Dmc1 and its cofactors (Table 1), meiosis-

Table 1 *Saccharomyces cerevisiae* proteins involved in meiotic recombination

Protein	Function
DSB formation/processing	
Spo11 ^a	DSB formation
Ski8/Rec103	Required for DSB formation; direct interaction with Spo11
Mei4 ^a	Required for DSB formation; subcomplex with Mer2/Rec107, Rec114
Mer2/Rec107 ^a	Required for DSB formation; subcomplex with Mei4, Rec114
Rec114 ^a	Required for DSB formation; subcomplex with Mei4, Mer2/Rec107; interaction with Rec102
Rec102 ^a	Required for DSB formation; subcomplex with Rec104 that interacts with Spo11
Rec104 ^a	Required for DSB formation; subcomplex with Rec102 that interacts with Spo11
Mre11-Rad50-Xrs2	Complex required for DSB formation and processing with DNA unwinding, DNA endonuclease and 3'-5' exonuclease, and DNA tethering activities (Xrs2 is NBS1 in mammals)
Sae2/Com1	ssDNA endonuclease, working in conjunction with MRX/N complex, required for DSB processing (mammalian homolog CtIP)
Exo1	5'-3' Exonuclease; possible role in resection of meiotic DSBs (see also postsynapsis)
Srs2	3'-5' DNA helicase with anti-recombination function; Rad51-ssDNA nucleoprotein filament disruption (similar function for mammalian BLM and RECQL5)
Rad51/Dmc1 filament formation	
RPA	Heterotrimeric single-stranded DNA binding protein, binds resected tails and likely displaced strand in D-loop, function in MMR
Rad51	Homology search and DNA strand exchange
Rad52	Mediator of Rad51, reannealing during second end capture and SDSA
Rad59	Reannealing during second end capture and SDSA?
Rad55-Rad57	Rad51 paralog complex, mediator of Rad51 (five mammalian Rad51 paralogs RAD51B, RAD51C, RAD51D, XRCC2, XRCC3)
Hed1 ^a	Meiosis-specific inhibitor of Rad51
Dmc1 ^a	Homology search and DNA strand exchange
Mei5-Sae3 ^a	Cofactor complex of Dmc1 (and possibly Rad51 in some organisms)
Mnd1-Hop2 ^a	Cofactor complex of Dmc1 (and possibly Rad51 in some organisms)
Rad54	Stabilization of Rad51 filament; enhances synapsis in Rad51-mediated in vitro recombination reactions
Rdh54/Tid1	Stabilization of Dmc1 filament; enhances synapsis in Rad51 (Dmc1?)-mediated in vitro recombination reactions

Table 1 (continued)

Protein	Function
Postsynapsis	
Rad54	Turnover of Rad51-dsDNA product complexes; branch migration?
Rdh54/Tid1	Turnover of Rad51/Dmc1-dsDNA product complexes; branch migration?
DNA polymerases and cofactors	Pol δ and possibly Pol λ are involved in DNA synthesis from invading 3' end; Pol δ is PCNA/RFC-dependent and the involvement of these cofactors is inferred; Pol δ /PCNA/RFC also function in MMR
Mer3 ^a	Helicase/DNA translocase with functions in heteroduplex DNA extension
Msh2	MutS homolog with functions in MMR and possibly heteroduplex rejection, in complexes with Msh3 and Msh6
Msh3	MutS homolog with functions in MMR and possibly heteroduplex rejection, in complexes with Msh2
Msh6	MutS homolog with functions in MMR and possibly heteroduplex rejection, in complexes with Msh2
Mlh1	MutL homolog with functions in MMR (as complexes with Mlh2, Mlh3, and Pms1) and CO promotion in the Msh4-Msh5 pathway (as complex with Mlh3)
Mlh2	MutL homolog with function in MMR in complex with Mlh1
Mlh3	MutL homolog with function in CO promotion in the Msh4-Msh5 pathway in complex with Mlh1
Pms1	MutL homolog with function in MMR and possibly heteroduplex rejection (note that mammalian homolog is called Pms2)
Rad1-Rad10	3' Flap endonuclease with function in repair of large insertion/deletion loops; possible function in removing 3' flaps resulting from excess DNA synthesis (XPF-ERCC1 in mammals)
Exo1	5'-3' Exonuclease, function in MMR and CO formation (see also presynapsis)
Msh4 ^a -Msh5 ^a	Complex with function in CO pathway 1
Mus81-Mms4	Complex with function in CO pathway 2 partially distinct from Msh4-Msh5 (Mms4 is Emel is fission yeast and mammals)
Sgs1-Top3-Rmi1	Complex with 3'-5' DNA helicase and type 1 topoisomerase activity, functions to dissolve joint molecules (analogous to BLM-TOPOIII α -BLAP75/RMI1 in mammals)
Srs2	3'-5' DNA helicase with anti-recombination function

^a The proteins are specifically expressed during meiosis in *S. cerevisiae* but not all are meiosis-specific in other organisms. Mer2/Rec107 protein is produced by meiosis-specific splicing involving the meiosis-specific splicing factors Mer1 and Mre2 (Engbrecht et al. 1991; Nakagawa and Ogawa 1997).

specific aspects of the S-phase preceding the first meiotic division (Cha et al. 2000; Watanabe et al. 2001), and meiosis-specific DNA checkpoint controls (Hochwagen and Amon 2006; Lydall et al. 1996). While many of the core recombination factors and meiosis-specific recombination proteins have been identified, our understanding of the mechanisms by which the recombination machinery is altered to accommodate the specific biological challenges of meiosis is still rather rudimentary. This review focuses on the biochemical properties of meiotic recombination proteins and is structured according to the mechanistic progression of meiotic recombination (Fig. 2).

2.1

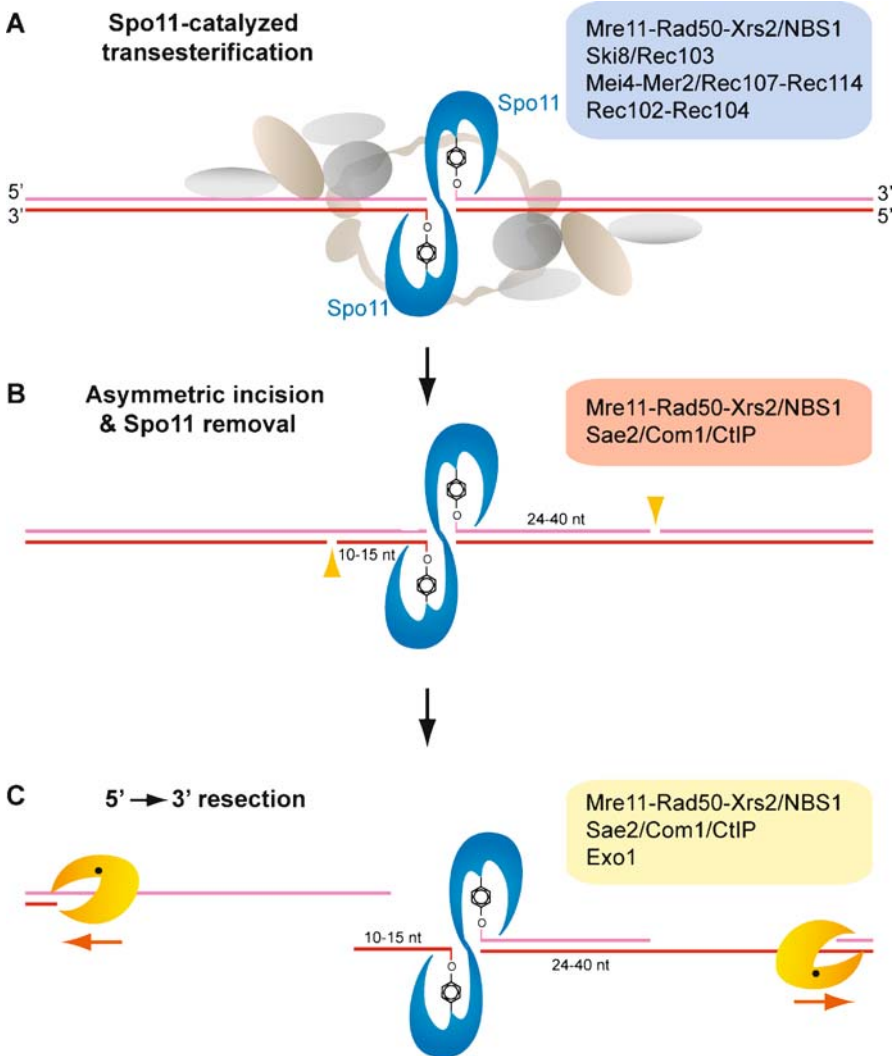
DSB Formation: Spo11 and its Control

The induction of meiotic recombination occurs largely through DSBs catalyzed by the Spo11 nuclease, a meiosis-specific protein with homology to the Top6A subunit of archaeal type IIB topoisomerases that is conserved in all eukaryotes (Bergerat et al. 1997) (see also S. Keeney, this SERIES, for a more detailed discussion). Spo11 functions similar to type II topoisomerases in that it forms a covalent intermediate between the active-site tyrosine and the 5'-end of the DSB, as deduced from *in vivo* studies (Fig. 3) (Keeney et al.

Fig. 3 Spo11-catalyzed DSBs and asymmetric end processing by 5' → 3' resection. **A** Spo11 is a type II-related topoisomerase that catalyzes DSB formation at “hotspots” by a transesterification mechanism involving a covalent intermediate between a tyrosine residue of a Spo11 subunit and each 5' end. A legion of factors (see *blue box*) is implicated in Spo11 DSB initiation, and their biochemical contributions to Spo11 activity need explanation. **B** Spo11 remains covalently bound to its product DNA break ends and can be isolated in two populations, bound to oligonucleotides of asymmetric lengths (Neale et al. 2005). The enzymes involved in the endonucleolytic incision are shown in the *red box*. One population is associated with short oligonucleotides, 10–15 nt, while a second population is recovered in association with oligonucleotides 24–40 nt in length. This result provides a possible mechanism to establish asymmetry of break ends at or near the timing of Spo11-catalyzed DSBs, although the underlying basis of the asymmetry is unknown. The two populations of oligonucleotide–Spo11 complexes imply that 5' → 3' resection initiates at nicks positioned asymmetrically to the Spo11 cleavage complex. **C** A number of factors are implicated in DNA end resection (see *yellow box*), but the primary exonuclease or endonuclease activities remain uncertain. Resection is processive up to ~500 nt on each break end and is possibly coupled to Rad51 and Dmc1 loading. The short oligonucleotide–Spo11 complex (10–15 nt) is suggested to separate readily from its complementary strand, generating a free 3' end (Neale et al. 2005). The longer oligonucleotide–Spo11 complex (24–40 nt) may remain paired to its complementary strand and therefore resection may generate a gapped region instead of a free end. These asymmetries may imply differential assembly of Rad51 and Dmc1 filaments (see Fig. 5). Alternatively, the oligonucleotides associated with Spo11 may remain base-paired to their complements, but the larger duplex extent on one side may present a binding site or interaction surface for a mediator protein specific for Dmc1 or Rad51 (see Fig. 4) ▶

1997). Mutational analysis (Spo11-Y135F in *S. cerevisiae*) demonstrated that the active site is essential for in vivo function (Bergerat et al. 1997). Unfortunately, the biochemistry of this initiation step is still lacking, due to the difficulty in purifying Spo11 protein and likely due to the complex control of Spo11 by at least nine other factors (see Table 1 and below) and the possible requirement for meiotic chromatin or chromosome structure.

Meiotic DSB formation by Spo11 depends in vivo on five meiosis-specific proteins (Mei4, Mer2/Rec107, Rec102, Rec104, Rec114) and Ski8/Rec103 protein, which exerts a dual function in RNA metabolism and meiotic recom-



bination (Fig. 3) (Arora et al. 2004). Extensive analyses have elucidated the physical and genetic interactions between these proteins (see Table 1) (Arora et al. 2004; Kee et al. 2004; Li et al. 2006). Ski8/Rec103 has the classical seven-bladed propeller structure of WD repeat proteins (Madrona and Wilson 2004; Seet et al. 2006). The WD repeat is a widely employed protein interaction motif, and Ski8/Rec103 appears to serve as a scaffold for the assembly of the Spo11 cleavage complex with direct interactions to Spo11 (Arora et al. 2004). Unfortunately, little is known about the biochemical activities of these proteins and therefore how they regulate or promote Spo11 DSB activity.

Meiotic DSB formation by Spo11 also depends on the Mre11-Rad50-Xrs2 (MRX) complex (Table 1), as no meiosis-specific DSBs are formed in deletion mutants of these three genes in budding yeast (NBS1 is the mammalian Xrs2 ortholog and the inclusive complex is referred to as MRX/N). The MRX/N complex exerts numerous functions in DNA damage checkpoints, mitotic DSB repair, and meiotic recombination (D'Amours and Jackson 2002; Keeney 2001). The strict dependency of Spo11 cleavage on the MRX complex is not conserved in *Arabidopsis* (Puizina et al. 2004) or in the fission yeast *S. pombe*, where meiotic DSBs are formed in the *rad50* and *rad32* (*mre11*) mutants with the proper timing, albeit at a reduced level compared to wild-type cells (Young et al. 2004). Since the MRX complex's conserved function in meiotic recombination appears to be DSB resection, the biochemical properties and cellular functions of this complex are discussed below (Sect. 2.2).

The biochemistry of the initiation of meiotic recombination and its control is shrouded in mystery. There is no mechanistic understanding to explain how Spo11 cleavage is targeted to a particular site. Moreover, it appears that Spo11 cleavage is restricted to a single sister chromatid in the bivalent (Fig. 1, part B), as the artificial *HIS4::LEU2* hotspot is cleaved with 25% efficiency, which is best explained by cleavage of a single sister chromatid in the bivalent in the absence of evidence for multiple cleavages (Hunter and Kleckner 2001). Lastly, it is unclear how Spo11 activity is restrained to make only a single cleavage per site during meiotic prophase. In analogy to DNA replication, where origin firing is limited to once per cell cycle, Spo11 cleavage may have a similar licensing requirement to limit its activity to once per meiosis at a given site (Blow and Laskey 1988).

2.2

Resection

Once generated by Spo11 and its associated factors, resection of the DSB proceeds in an apparent 5'-3' direction, resulting in the 3'-OH ending ssDNA tail needed for Rad51/Dmc1 filament formation and DNA strand invasion (Figs. 2, 3). Mechanistically, resection could be achieved by a 5'-3' dsDNA exonuclease, a 5'-3' ssDNA exonuclease in combination with a DNA helicase, or by an ssDNA endonuclease in combination with a DNA helicase. In vivo data

using separation of function, non-null mutations in *RAD50* and *MRE11* implicated the MRX/N complex in DSB resection. Such *rad50-s* mutations map near to the Walker A box ATP binding consensus sequence (Alani et al. 1990). Mutants in the phosphoesterase motif of *MRE11* that eliminated all its nuclease activities in vitro displayed the same phenotype as *rad50s* mutants by accumulating unresected meiotic DSBs with 5' ends covalently attached to the Spo11 protein (Keeney et al. 1997; Moreau et al. 1999; Nairz and Klein 1997; Tsubouchi and Ogawa 1998; Usui et al. 1998). This key result not only demonstrated that Spo11 delivers the meiotic DSB, but it also identified a post-DSB role for the MRX/N complex in meiotic recombination. How does the MRX/N complex function during DSB resection and what other enzymes are involved in this process?

2.2.1

The Multifunctional MRX/N Complex

The MRX/N complex is a multiprotein assembly with several functions in DNA damage signaling, NHEJ, and recombination (Hopfner and Tainer 2003; Krogh and Symington 2004). MRX/N is one of the first protein complexes found at DSBs and a primary sensor for the activation of the Mec1/Tel1 signaling pathways in *S. cerevisiae*. However, this signaling role and the effector pathways controlled by it, as well as its role in NHEJ, will not be further discussed here (for reviews see D'Amours and Jackson 2002; Stracker et al. 2004).

Mre11 has an N-terminal phosphoesterase motif that provides the single catalytic center for all its nuclease activities (see below). This nuclease function is essential for Mre11 in meiosis, but in vegetative (somatic) cells, the phenotypes of nuclease-deficient Mre11 mutants are far less severe than those of null mutants (Moreau et al. 1999). In addition to its nuclease domain required for DSB processing in meiosis, Mre11 also contains two DNA binding domains in the C-terminal half of the protein, which are required for meiotic DSB formation (Furuse et al. 1998). Rad50 is an SMC-type (*structural maintenance of chromosomes*) protein with an N-terminal Walker A and a C-terminal Walker B box that compose an intramolecular ATPase domain and a central extended intramolecular coiled-coil domain (de Jager et al. 2001b). The Rad50 ATPase activity is essential for all functions of the MRX complex in vivo (Alani et al. 1990). Binding of ATP or non-hydrolyzable ATP analogs stimulates Rad50 DNA binding by inducing dimerization (Hopfner et al. 2000), suggesting that DNA binding is regulated by the nucleotide cofactor cycle. The ATPase domain of Rad50 is related to the ABC transporter ATPases (Hopfner and Tainer 2003), which have been shown to share structural similarity with adenylate kinases. In fact, human and yeast Mre11-Rad50 complexes display adenylate kinase activity ($\text{ATP} + \text{AMP} \leftrightarrow \text{ADP} + \text{ADP}$) (Bhaskara et al. 2007), but it is unclear how this activity impacts the biochemical and cellular functions of the MRX/N complex. Two Mre11 subunits

interact with the ATPase heads of a Rad50 dimer, forming a hetero-tetramer. The Xrs2/NBS1 subunit lacks apparent enzymatic activity and associates with the Mre11-Rad50 assembly by binding to Mre11 through a C-terminal binding site (Shima et al. 2005). Key features of Xrs2/NBS1 are an FHA and a tandem BRCT domain, which are known phospho-specific protein interaction modules (Seet et al. 2006). These domains probably confer regulated protein interactions on the MRX/N complex, but the target proteins during meiotic recombination have not been identified yet.

The biochemical properties of Mre11-Rad50 and/or MRX/N complexes are expansive and include nuclease, DNA unwinding, DNA annealing, and DNA tethering activities *in vitro* (for review see D'Amours and Jackson 2002; Krogh and Symington 2004). Mre11 exhibits Mn^{2+} -dependent 3'-5' dsDNA exonuclease activity, as well as ssDNA and dsDNA endonuclease activities, which are enhanced by the presence of Rad50 and Xrs2/NBS1 (Furuse et al. 1998; Paull and Gellert 1998; Trujillo et al. 1998; Usui et al. 1998). The ssDNA endonuclease activity appears responsible for the processing of a covalent protein-DNA intermediate defined by Spo11 bound to the 5'-end of the DSB; Mre11 ssDNA endonuclease activity releases Spo11 as an oligonucleotide-bound form (Neale et al. 2005) (Fig. 3). Spo11 removal may in fact be the essential function of Mre11 in meiosis. This might explain why Mre11 nuclease-deficient mutants display a much more severe phenotype in meiotic cells than in vegetative cells, if further break end processing requires nucleolytic removal of the protein that catalyzed the DSB. Furthermore, an unexpected asymmetry in the physical properties of the break ends on either side of the Spo11 cleavage may lead to a distinction between the two ends, and mechanistic implications are discussed later (see Fig. 3). The nucleolytic release of Spo11 by the MRX/N endonuclease activity furthermore requires an associated unwinding activity, which may also be supplied by the MRX/N complex, as the human MRN complex displays weak strand dissociation activity (Paull and Gellert 1999). The unwinding activity is stimulated by, but is not dependent on, ATP. This feature and the absence of a motor domain found in traditional DNA helicases makes it unlikely that MRX/N functions by translocating on ssDNA, and instead suggests a stoichiometric mechanism of binding to ssDNA akin to ssDNA binding proteins. Beyond a contribution to Spo11 release, the unwinding activity might also be involved in further processing of the DSB, although there is no direct evidence for this at present. It is also possible that a DNA helicase cooperates with the MRX/N complex in this function. Finally, human Mre11 was also found to reanneal complementary ssDNA *in vitro*, an activity that was abrogated when RPA was bound to ssDNA (de Jager et al. 2001a). The biological significance of this biochemical activity is unclear.

The DNA tethering activity of the MRX/N complex is of particular interest, and led to the elegant suggestion that the MRX/N complex functions like "molecular Velcro" to coordinate the two ends of a DSB or two DNA

molecules (de Jager et al. 2001b). The coiled-coil domain of Rad50 folds into a 50 nm long stalk protruding from the ATPase head domain. The apex of the coiled-coil contains a zinc-hook, which can non-covalently link two Rad50 coiled-coil domains by shared binding of a zinc atom (Hopfner et al. 2002). The coiled-coil domains are rather flexible in solution, leading to the possibility of an intramolecular connection between the two Rad50 coiled-coils within a single MRX/N assembly. Such an intramolecular interaction would frustrate intermolecular interactions between MRX/N assemblies needed for DNA tethering. Direct observation by atomic force microscopy provided a solution to this conundrum, demonstrating that DNA binding by MRN stiffens the coiled-coil, which will prevent intramolecular interactions and favor the intermolecular associations needed for tethering (Moreno-Herrero et al. 2005). The Rad50 zinc-hook mutant in budding yeast is deficient in meiotic DSB formation (Wiltzius et al. 2005), suggesting a function of DNA tethering by MRX in this process, either by coordinating the recombining homologs or by putting in place a tether that bridges the future DSB.

In sum, biochemical analysis of the MRX/N complex and the genetic analysis of MRX mutants in yeast have provided evidence for multiple functions of MRX/N in meiosis. The DNA tethering function is important for Spo11-dependent DSB formation, but it is unclear at the moment whether MRX/N also coordinates the two DSB ends of the meiotic DSB. In addition, the MRX/N ssDNA endonuclease activity is critical for initial processing of the covalent Spo11-DNA intermediate, releasing a Spo11-oligonucleotide complex, and may also be involved in further resection of the DSB. An important but not essential role of the MRX complex in post-DSB events during meiotic recombination is indicated by results from inducing meiotic DSBs by meiosis-specific expression of the HO-endonuclease in a *rad50* deletion strain that is deficient in Spo11-mediated DSB formation (Malkova et al. 1996). While recombination was induced by the meiotic HO-mediated DSBs, some DSBs were not repaired, suggesting that Rad50 and by implication the MRX complex are required downstream of Spo11 removal from meiotic DSBs.

2.2.2

Sae2/Com1

SAE2 (a.k.a. *COM1*) was identified in genetic screens for mutations with the meiotic phenotype of *rad50-s* mutants (McKee and Kleckner 1997a; Prinz et al. 1997). Like *rad50-s* (or *mre11-s*) mutations, null mutants of *SAE2* accumulate unresected meiotic DSBs. Sae2 exhibits ssDNA endonuclease activity, and cooperatively cleaves hairpin structures in the presence of the MRX complex (Lengsfeld et al. 2007). Unlike MRX, Sae2 is not involved in DSB formation. Moreover, meiosis-specific expression of the VDE-endonuclease in a Spo13-deficient yeast strain demonstrated that DSBs are processed in the absence of Sae2 (Neale et al. 2002). These results suggest that Sae2 spe-

cifically collaborates with the MRX complex in the release of Spo11 from the break during meiosis. Sae2 is also a phosphorylation substrate for the Mec1/Tel1 kinases during meiosis (Cartagena-Lirola et al. 2006). Sae2 phosphorylation site mutants display a defect in meiotic DSB end-processing (Cartagena-Lirola et al. 2006) but normal catalytic activity (Lengsfeld et al. 2007), leaving open the in vivo function of Sae2 phosphorylation. Sae2 is conserved in eukaryotes and is called CtIP in mammals (Penkner et al. 2007; Sartori et al. 2007; Uanschou et al. 2007). The meiotic phenotypes of Sae2 mutants in *Arabidopsis* and *C. elegans* are consistent with a role in meiotic DSB end processing (Penkner et al. 2007; Uanschou et al. 2007). In sum, the processing of Spo11-mediated DSBs requires at least two nucleases, the MRX complex and Sae2. While it is clear that the Mre11 nuclease activity is essential for Spo11 removal, it needs to be established whether this also holds for the Sae2 nuclease activity or whether Sae2 acts as an MRX/N co-factor during this process. The biochemical details of how these proteins interact and cooperate can now be defined, having the proteins available, but will require the cognate substrate of Spo11 covalently attached to 5' ends of a DSB.

2.2.3

Exo1

Exo1 is a 5'-3' exonuclease of the Rad2 family, first identified by a biochemical approach in meiotic *S. pombe* cells. In *S. pombe*, Exo1 activity is induced in meiosis (Szankasi and Smith 1992). Exo1 has been implicated at multiple stages during meiotic recombination, including end-resection, MMR, and CO control (Tran et al. 2004). In budding yeast, *exo1-Δ* mutants exhibit spore viability slightly decreased from wild-type (79% relative to 98%) (Khazanehdari and Borts 2000), with a pattern of increased two- or zero-viable spore tetrads consistent with meiosis I non-disjunction. CO is reduced approximately twofold, and is associated with shorter gene conversion tract lengths (Khazanehdari and Borts 2000). In *Exo1*^{-/-} mutant mice, homolog pairing and synaptonemal complex formation are normal, but COs are severely reduced and resemble levels in *Mlh1*^{-/-} and *Mlh3*^{-/-} mice (Wei et al. 2003) (see Sect. 2.9.3). Exo1 displays non-processive 5'-3' exonuclease activity with a twofold preference for dsDNA over ssDNA (Fiorentini et al. 1997). This activity depends on a divalent cation and can use Mg²⁺ or Mn²⁺ with almost equal efficiency. The exonucleolytic properties of Exo1 are suited for continued resection at meiotic DSBs after the initial processing by MRX/N-Sae2. However, genetic analysis has shown that meiotic DSBs are resected in *exo1* mutants; nevertheless, the hyper-resection seen in meiotic mutants with DNA pairing defects (for example *dmc1*, see below) is decreased in Exo1-deficient cells (Tsubouchi and Ogawa 2000). Hence, it is possible that Exo1 contributes to 5'-3' processing after Spo11 turnover by MRX/N-Sae2 in wild-type cells,

but other activities may compensate in its absence. It is unclear whether the effect of the *exo1* mutants on CO is an indirect consequence of a resection defect or indicative of an additional subsequent role of the protein in CO formation (see Sect. 2.9).

2.3

Rad51/Dmc1 Filament Formation

2.3.1

The Homology Search and DNA Strand Exchange Protein Rad51

Rad51 is the evolutionarily conserved RecA homolog found in all eukaryotes and performs the central aspect of recombination: homology search and DNA strand invasion (Aboussekhra et al. 1992; Bianco et al. 1998; Shinohara et al. 1992; Sung 1994). Rad51 is essential for mitotic and meiotic recombination (Hunter 2007; Krogh and Symington 2004; Paques and Haber 1999). *rad51-Δ* mutants in budding yeast show nearly complete meiotic failure; the few spores formed (on the order of 1% relative to 80–90% in wild-type) are inviable. Meiotic cells accumulate hyper-resected DSBs and exhibit a reduced yield of physical recombinants (Shinohara et al. 1992). Most relevant to the role of *RAD51* in promoting the meiotic agenda of regulated interhomolog exchange is the observation that the interhomolog bias is lost in the *rad51-Δ* mutant; the ratio of interhomolog to intersister joint molecules is reduced by 7.3-fold (from ~2.4 in wild-type to 0.33 in *rad51-Δ*) (Schwacha and Kleckner 1997). Any model for meiotic recombination must therefore account for the role of Rad51 in promoting interhomolog joint molecules.

S. cerevisiae Rad51 protein forms a right-handed filament with a helical pitch of 130 Å on dsDNA and ssDNA, as determined by crystallographic and electron microscopic studies (Conway et al. 2004; Ogawa et al. 1993; Yu et al. 2001). Binding of ATP induces a high-affinity DNA binding state in Rad51, and ATP hydrolysis lowers this affinity. This effectively links DNA binding with the nucleotide cofactor cycle, although there are organism-specific variations in how nucleotide-cofactor regulates Rad51 DNA binding (for a discussion see Heyer 2007). As for all filament-forming proteins, including RecA, nucleation of the Rad51 filament (binding of the first subunit(s) to DNA) is the rate-limiting step, suggesting the need for cofactors that are functionally equivalent to bacterial RecFOR and RecBCD that load RecA on ssDNA (Bianco et al. 1998). Unlike RecA, which displays a kinetic delay binding to dsDNA, effectively targeting the protein to ssDNA, Rad51 has little preference for ssDNA over dsDNA (Bianco et al. 1998; Zaitseva et al. 1999). This opens a question of how Rad51 is targeted to ssDNA to form the presynaptic filament, which is key for homology search and DNA strand invasion. Compared to RecA, Rad51 displays a significantly lower (over 100-fold reduced) ATPase activity, particularly on dsDNA (Bianco et al. 1998). This lower ATPase activ-

ity leads to reduced dynamics of the Rad51-dsDNA filament in turning over and releasing bound DNA. The biochemical differences between RecA and Rad51 suggest that Rad51 needs additional cofactors (mediators) for the assembly of a dynamic and functional Rad51 presynaptic filament and for the dissociation of Rad51-dsDNA complexes. This is true for Rad51 in both vegetative and meiotic cells, although genetic evidence indicates certain differences in mediator requirement between meiotic and mitotic recombination. Furthermore, meiotic recombination uses Rad51 in a highly coordinated series of events that feature its meiosis-specific paralog, Dmc1 (see Sect. 2.3.4). This invokes meiosis-specific regulation of Rad51.

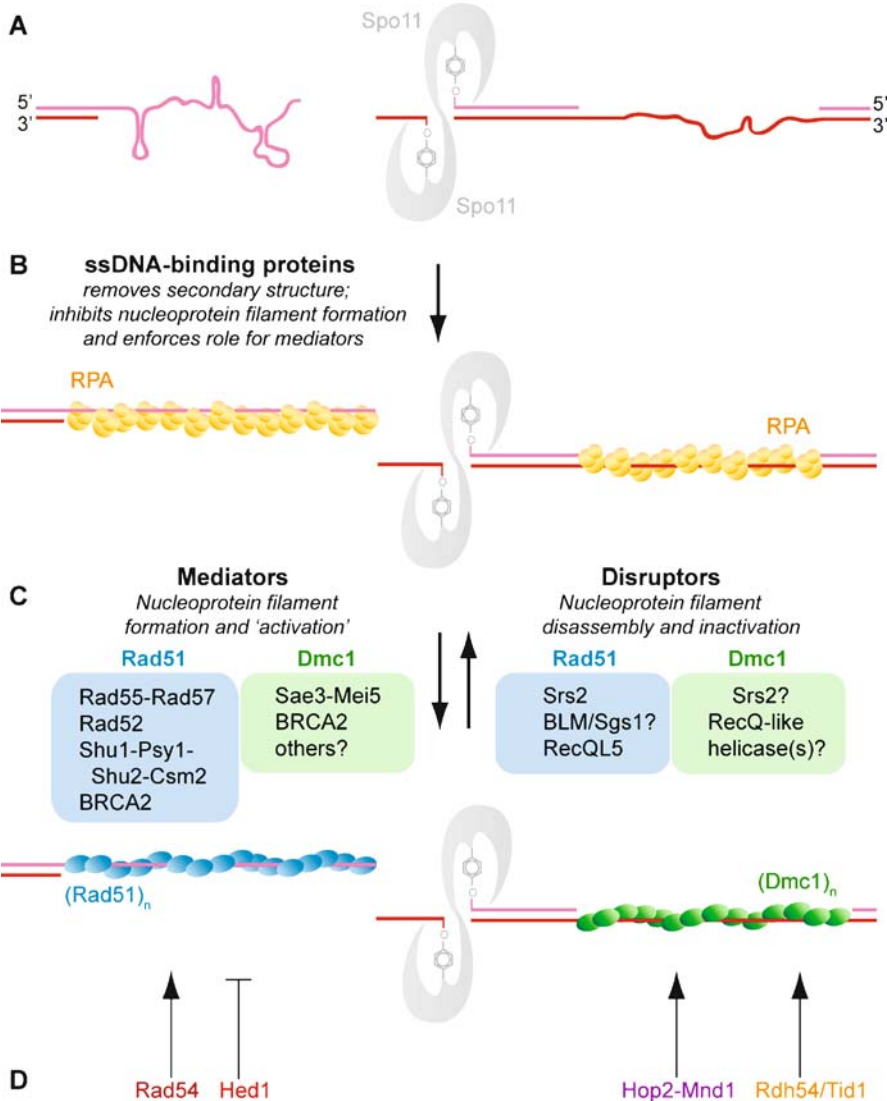
2.3.2

Rad52, Rad55-Rad57, RPA: Mediators of Rad51 Filament Assembly on ssDNA

At least three distinct cofactors help in the assembly of Rad51 on ssDNA to form the presynaptic filament in mitotic and meiotic recombination: the heterotrimeric ssDNA binding protein RPA, the heterodimer of the Rad51 paralogs Rad55-Rad57, and the Rad52 protein (Fig. 4).

Fig. 4 Regulation of Rad51 and Dmc1 nucleoprotein filament formation and function. ►
A The ssDNA generated by 5' → 3' resection is likely to form secondary structures inhibitory to the formation of active Rad51 or Dmc1 nucleoprotein filaments. **B** RPA overcomes this challenge to Rad51 or Dmc1 by melting ssDNA secondary structure during binding, but RPA binds avidly to ssDNA and therefore inhibits nucleoprotein filament formation because RPA is poorly displaced by Rad51 or Dmc1 alone. **C** Aside from indirectly promoting nucleoprotein filament formation on ssDNA, RPA presents an opportunity for regulation of Rad51 or Dmc1 loading on ssDNA by enforcing a role for mediators. Mediators are a class of proteins that can best be characterized as factors that promote functional filaments (assayed by capacity for DNA strand exchange), although the mechanisms by which they promote a functional filament may be diverse and include: (1) regulation of RPA displacement and Rad51 nucleation on ssDNA, (2) regulation of Rad51 stability (turnover rates) on ssDNA, at a dsDNA-ssDNA junction, or on heteroduplex DNA, (3) filament nucleation and regulation of filament growth, or (4) a function with the free subunit pool. The asymmetry of Spo11 cleavage complexes may suggest a role for mediators specific to nucleation of Rad51 or Dmc1 at one or the other face of the cleavage complex. In some contexts, Srs2 may be considered a mediator of functional filaments if its displacement of Rad51 allows proper registry of a contiguous filament rather than small Rad51 patches on a ssDNA lattice that may be out of register from one another. Biochemically, Srs2 removes Rad51 from ssDNA; Dmc1 remains to be tested. **D** Other factors further regulate the function of an assembled Rad51-ssDNA or Dmc1-ssDNA filament. Rad54 stabilizes the Rad51-ssDNA filament and promotes the DNA strand exchange activity of Rad51 filaments by mechanisms that may include topological remodeling of the dsDNA target. Rdh54/Tid1 also stimulates Rad51 DNA strand exchange (Petukhova et al. 2000), and by analogy, Rdh54/Tid1 likely interacts with the Dmc1 filament to promote its DNA strand exchange activity. Genetically, Hed1 appears to inhibit DNA strand exchange activity catalyzed by the Rad51 filament, but biochemical data is lacking. Hop2-Mnd1 promotes duplex capture by the Dmc1 filament

RPA has multiple functions during recombination and is generally expected to be the first protein to access ssDNA generated in vivo. First, in Rad51 filament assembly, RPA is critical to counteract secondary structure in ssDNA. Secondary structure in the ssDNA would interrupt the formation of functional Rad51 presynaptic filaments, because Rad51 also binds dsDNA. Secondly, RPA binds the displaced strand and consequently stabilizes this intermediate (Eggler et al. 2002). In vitro, Rad51 is strongly stimulated by substoichiometric RPA in reactions with ssDNA that has the po-



tential to form secondary structure, but not with ssDNA devoid of secondary structure (Sugiyama et al. 1997; Sung 1994). However, because RPA displays much higher affinity to ssDNA than Rad51, Rad51 is extremely slow to form filaments on ssDNA precoated with RPA, leading to inhibition of Rad51-mediated DNA strand exchange when RPA-coated ssDNA is used. This challenge to Rad51 filament nucleation and propagation is managed by a class of proteins called mediators. The ability of the recombination mediator proteins Rad52 and Rad55-Rad57 to allow Rad51-mediated DNA strand exchange with RPA-coated ssDNA has been demonstrated in vitro (New et al. 1998; Shinohara and Ogawa 1998; Sung 1997a,b). Furthermore, in vivo Rad51 filament formation in meiosis depends on Rad52 and Rad55-Rad57, consistent with the biochemical data (Gasior et al. 1998). Rad51 filament formation is assessed by the formation of transient, Spo11-dependent immunostaining foci that form during meiotic prophase (Bishop 1994). These foci likely represent the Rad51 presynaptic filament and Rad51-mediated pairing intermediates, and their dependence on Rad52 and Rad55-Rad57 indicates that functional Rad51 filaments require mediator contributions. What are the biochemical properties of these mediators and the mechanisms involved in Rad51 nucleoprotein filament promotion?

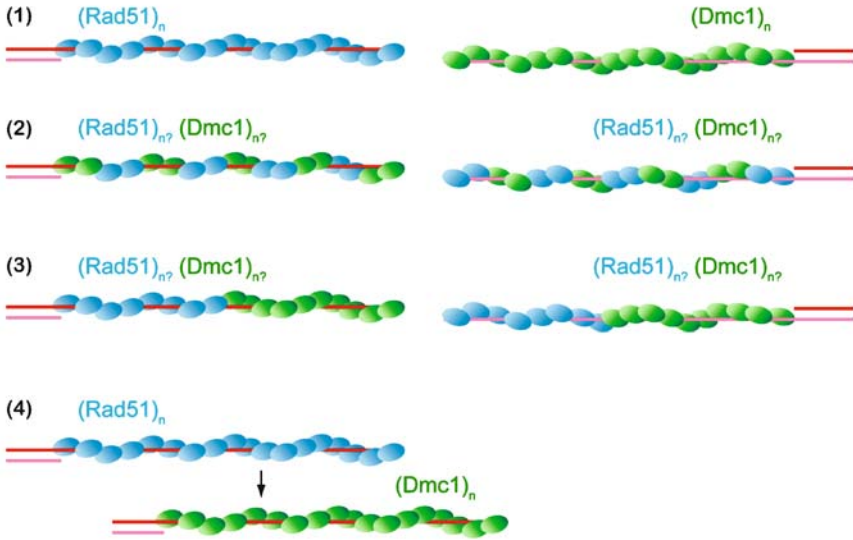
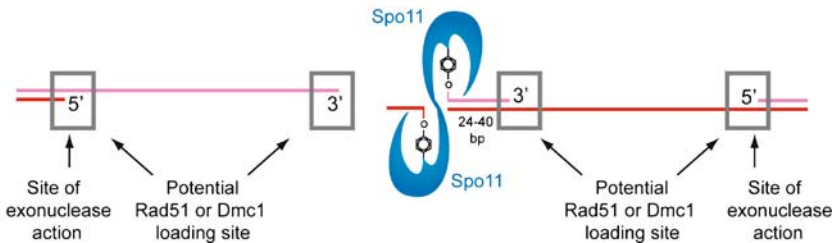
Rad52 appears to be important to all applications of Rad51-mediated DNA strand exchange. In *rad52-Δ* mutants in budding yeast, Rad51 foci fail to appear and the few spores that form are inviable (< 1% viability) (Borts et al. 1986; Gasior et al. 1998; Resnick et al. 1986). The frequency of CO recombinants in surviving spores is reduced by 100- to 1000-fold relative to wild-type levels (Borts et al. 1986), and the true reduction is probably even greater than this value, because most of the recombinants scored in surviving spores may in fact represent half-crossovers (non-reciprocal events that yield apparent crossovers but are in fact the result of pathological events). Despite the failure to form Rad51 foci, Dmc1 filaments must be somewhat functional in *rad52-Δ* mutants; physical analysis of recombination intermediates in *rad52-Δ* cells shows that SEIs reach wild-type levels, but interhomolog dHJs are reduced eightfold (Lao et al. 2007). These events are very likely mediated by Dmc1, because of the mediator defect of *rad52* mutants. Furthermore, recombination in *rad52-Δ* single mutants is indistinguishable from recombination in *rad52-Δ dmc1-Δ*, i.e., nearly completely abolished. The apparent epistatic relationship between *RAD51* and *RAD52* in meiotic recombination, as assayed by the null alleles, conceals a later role for Rad52. However, analysis of the *rad52-327* allele that encodes a protein defective in physical interaction with Rad51, demonstrated a role of Rad52 in second-end capture during DSBR (Lao et al. 2007) (see Sect. 2.8).

Rad52 protein has a conserved N-terminal DNA binding domain and forms a multimeric ring-shaped structure that binds ssDNA on the outside face of the ring (Kagawa et al. 2002; Shinohara et al. 1998; Singleton et al. 2002; Stasiak et al. 2000). *S. cerevisiae* Rad52 binds to Rad51 through a C-terminal binding

domain, and also interacts directly with RPA, based on genetic, cytological, and two-hybrid data (Firmenich et al. 1995; Gasior et al. 1998; Hays et al. 1998; Krejci et al. 2002). Rad52 is critical for the ejection of RPA upon Rad51 binding to ssDNA (Sugiyama and Kowalczykowski 2002). In analogy to the related T4 UvsY protein (Beernink and Morrical 1999), one could envision that Rad52 kinks the RPA-coated ssDNA template to allow nucleation of the Rad51 filament. In vegetative cells, the recombination defect of budding yeast *rad52* mutants is much more extreme than that of *rad55* (or *rad57*) mutants and also than that of *rad51* mutants, because of the multiple roles of Rad52 in HR (Rad51 filament formation discussed here, strand annealing in DSBR and SDSA discussed below) as well as in SSA (Krogh and Symington 2004; Paques and Haber 1999). However, a tight requirement for both Rad52 and Rad55-Rad57 is suggested for the meiotic setting (Gasior et al. 2001, 1998). In summary, Rad52 plays a significant role in Rad51 filament formation, likely by facilitating the nucleation of Rad51 filaments on RPA-coated ssDNA. How its mediator function intersects with Rad55-Rad57 mediator function to accomplish meiotic Rad51 filament assembly remains to be defined. It should be noted that Rad52 protein does not exert an equally important role in HR in vertebrates, as the respective mutants in mice display very mild phenotypes (Rijkers et al. 1998). It is unclear which other protein(s) have usurped Rad52 functions.

Rad55/57

Rad55 and Rad57 are Rad51 paralogs and share with Rad51 the RecA core, which comprises the ATPase domain (Krogh and Symington 2004; Paques and Haber 1999). They form a heterodimer that displays ATPase activity, but is unable to perform strand invasion reactions (Sung 1997b). While Rad55-Rad57 is not essential for Rad51 focus formation after ionizing radiation in mitotic cells (Lisby et al. 2004), it is absolutely required for meiotic Rad51 focus formation in vivo (Gasior et al. 1998). *rad57-Δ* mutants in budding yeast resemble *rad52-Δ* mutants for their meiotic phenotypes, but may yield slightly higher viable spores (< 10% viable) (Borts et al. 1986). In essence, *rad51*, *rad57*, and *rad55* mutants are identical at the level of meiotic phenotype defined by joint molecule yields in the physical analysis of recombination intermediates. Like *RAD51*, *RAD55* and *RAD57* are required for the interhomolog bias observed in budding yeast meiotic recombination (Schwacha and Kleckner 1997), likely because of their Rad51 mediator role. Rad55-Rad57 interacts with Rad51 protein (Sung 1997b), but in contrast to Rad52, no interaction with RPA has been reported. Yet, Rad55-Rad57 is needed for Rad51 DNA strand exchange activity with RPA-coated ssDNA in vitro (Sung 1997b), although the mechanisms involved are unclear. Rad55-Rad57 might be a nucleating factor as proposed for Rad52. Alternatively, Rad55-Rad57 might also stabilize Rad51 filaments or short Rad51 patches that lead to formation of a longer filament. It is likely that Rad52 and Rad55-Rad57 play distinct, non-

A vegetative DSB**B Spo11-induced (meiotic) DSB****C**

overlapping roles in Rad51 filament formation, since both are required for focus formation during meiosis *in vivo* (Gasior et al. 1998). Biochemical analysis of Rad51-mediated recombination reactions in the presence of RPA and both mediators (Rad52, Rad55-Rad57) should provide some insights. While there is no functional equivalent of the RecBCD complex in eukaryotes, the bacterial RecFOR complex that targets RecA filament formation to a ssDNA-dsDNA junction might serve as a useful paradigm (Morimatsu and Kowalczykowski 2003). Such a model would predict a competition between resection and filament nucleation, as both processes act on the same intermediate (see Fig. 5, part C).

- ◀ **Fig. 5** Models for Dmc1 and Rad51-induced DSB ends: cofilaments or asymmetric filaments. **A** Rad51 is the sole RecA homolog employed during vegetative recombination in *S. cerevisiae*. It assembles as symmetric filaments on each DSB end, although each end may be differentially regulated (for DNA strand exchange or second-end capture in DSBR) and these details await biochemical explanation. **B** Dmc1 is a meiosis-specific Rad51 paralog that functions in collaboration with Rad51 for the purposes of homolog-directed DNA strand exchange with resolution to CO. There are at least four possibilities for the collaborative relationship of Rad1 and Dmc1 in filaments: 1 Rad51 and Dmc1 may assemble as separate filaments on each break end; 2 Rad51 and Dmc1 may assemble as mixed filaments on each break end; 3 Rad51 and Dmc1 may assemble as patchy cofilaments on each break end; or 4 Rad51 and Dmc1 may assemble consecutively on the same ssDNA regions during different stages of meiotic recombination. **C** The asymmetry suggested for Spo11-induced DSB processing (Neale et al. 2005) presents several opportunities to direct different loading of Rad51 and Dmc1 to one or the other break end. Furthermore, the break ends may remain associated but the different oligo lengths adjacent to the Spo11 homodimer may present binding sites for recruitment of Rad51- or Dmc1-specific mediators

The Shu1-Psy1-Shu2-Csm2 complex in budding yeast (Shor et al. 2005) might represent yet another Rad51 cofactor complex (reviewed in Heyer 2007). The similarity of Shu1 and Psy3 with distant Rad51 paralogs (fission yeast Rlp1/mammalian Xrcc2 and fission yeast Rdl1/mammalian Rad51D, respectively) and the known function of the fission yeast and mammalian proteins in Rad51 focus formation in vivo (Haruta et al. 2006; Martin et al. 2006) provides motivation for further genetic and biochemical studies.

2.3.3

Hed1: A Meiosis-Specific Rad51 Inhibitor

In its fundamental properties, Rad51 filament assembly in meiotic cells may closely resemble filament assembly in vegetative cells, but meiotic recombination appears to invoke a unique mode for the temporal or physical control of subsequent Rad51 DNA strand exchange activity. Hed1 is an unusual meiosis-specific protein that appears to antagonize the function of Rad51 protein (Tsubouchi and Roeder 2006). Since Rad51 is required for meiotic recombination, it has been speculated that Hed1 coordinates Rad51 and Dmc1 function. Hed1 colocalizes with Rad51 at meiotic DSBs, and the dependence of this Hed1 localization on Rad51 (Tsubouchi and Roeder 2006) suggests that Hed1 functions after assembly of the Rad51 filament (Fig. 4, part D). Hed1 inhibition of Rad51 function can be overcome by increased levels of Rad51 or Rad54, at least in Dmc1-deficient cells, where overexpression of these proteins suppresses the meiotic arrest and DSB repair defects of *dmc1* mutants (Shinohara et al. 2003; Tsubouchi and Roeder 2003). This suggests that Hed1 might affect the Rad51-Rad54 interaction. It will be of interest to determine the mechanism of Hed1 inhibition of Rad51-mediated recombination. Since Rad51 is required for meiosis, the Hed1 inhibition of its function is likely to be transient.

2.3.4

Dmc1: The Meiosis-Specific RecA Homolog

Key to the fundamental outcome of meiotic recombination is the meiosis-specific expression of additional recombination factors that target DNA strand exchange to the homologs, at the relative exclusion of the sister chromatid. Meiotic homolog bias in most eukaryotes apparently cannot be achieved by modulation of Rad51 alone. The meiosis-specific recombination factors therefore include the Rad51 paralog, Dmc1, and its accessory factors, the Mei5-Sae3 and Hop2-Mnd1 complexes (Fig. 4). *DMC1* was identified in a screen for meiosis-specific transcripts and is essential for interhomolog recombination during meiosis (Bishop et al. 1992; Hunter and Kleckner 2001; Schwacha and Kleckner 1997). The fundamental role for Dmc1 in directing meiotic recombination to homologs is inferred by the spore inviability of *dmc1* mutants and the absence of junction intermediates (SEI, dHJ) (Schwacha and Kleckner 1997), leading to a dramatic decrease in CO formation (10–30% of wild-type levels) (Bishop et al. 1992; Rockmill and Roeder 1994; Rockmill et al. 1995). Like *rad51-Δ* mutants, *dmc1-Δ* mutants accumulate hyper-resected DSBs. Also like *rad51-Δ* mutants, *dmc1-Δ* mutants in budding yeast show low spore formation (1% relative to 79% in wild-type) and low spore viability (below 2.5% relative to 94% in wild-type) (Bishop et al. 1992). The poor spore formation is probably explained by arrest in meiotic prophase in certain strain backgrounds.

Dmc1 is a RecA homolog with unique N- and C-terminal extensions, and it forms the typical helical filament on DNA and performs DNA pairing reactions in vitro. The conditions for Dmc1 filament formation are more narrow than for Rad51 or RecA, which led to initial difficulties to develop robust assays for this protein (reviewed in Neale and Keeney 2006). The specific conditions that allow filament formation and more efficient recombination by Dmc1 (and also human Rad51) include high levels of Ca^{2+} or increased salt concentrations (Bugreev et al. 2005; Lee et al. 2005; Sauvageau et al. 2005; Sehorn et al. 2004). The free intracellular (cytosolic) Ca^{2+} concentration is less than 1 μM in mammals, i.e., substantially below the 100–400 μM concentration required for optimal in vitro stimulation (Bugreev et al. 2005). One could surmise that these experimental conditions substitute for cofactors found in vivo.

Similar to RecA and Rad51, nucleation of the Dmc1 filament is expected to be rate-limiting, in particular on RPA-coated ssDNA. The Sae3-Mei5 complex is a Dmc1-specific cofactor and may function in this mediator context (see below). Like Rad51, Dmc1 displays low ATPase activity compared to RecA and displays little preference for ssDNA over dsDNA (Hong et al. 2001; Li et al. 1997). These biochemical properties necessitate a factor that dissociates Dmc1-dsDNA complexes, and Rdh54/Tid1 appears to be specific for Dmc1 in this respect (Holzen et al. 2006) (see below).

It has been noted that organisms that lack Dmc1 (and Hop2-Mnd1) rely on specific pairing sites that mediate meiotic homolog pairing (such as *Drosophila* or *C. elegans*), while organisms that employ these proteins lack such pairing sites (such as budding yeast, mammals). This may suggest that Dmc1 and its cofactors are specifically involved in establishing homolog interactions (Stahl et al. 2004; Villeneuve and Hillers 2001), but a biochemical explanation for the role of Dmc1 in promoting homolog bias is still needed.

2.3.5

Sae3-Mei5: A Meiosis-Specific Mediator Complex for Dmc1

Sae3 and Mei5 form a meiosis-specific complex in budding yeast that is required for Dmc1 focus formation *in vivo* (Hayase et al. 2004). Cytological and genetic analyses suggest that the mediator role of Mei5-Sae3 is specific for Dmc1 filament formation, at least in budding yeast (Hayase et al. 2004; McKee and Kleckner 1997b; Tsubouchi and Roeder 2004) (Fig. 4). Like *rad51-Δ*, *dmc1-Δ*, *rad55-Δ*, *rad57-Δ* and *rad52-Δ* mutants, *sae3-Δ* mutants accumulate hyper-resected DSBs. More specifically, *sae3-Δ* mutants are nearly indistinguishable from *dmc1-Δ* mutants in their arrest at pachytene, reduced sporulation and spore viability levels, and reduced crossover levels (at 15% recombinant products as opposed to 80% in wild-type cells). As for the relationship of Rad51 and its mediators (Rad52, Rad55-Rad57), *sae3-Δ* and *dmc1-Δ* are fully epistatic. In *S. cerevisiae*, mutations in *SAE3* and *MEI5* do not affect Rad51 filament formation (Hayase et al. 2004). Dmc1 foci, however, depend on Sae3-Mei5 and the localization of Sae3-Mei5 in turn depends on Dmc1, suggesting mutually dependent localization (Tsubouchi and Roeder 2004).

While the biochemistry of the budding yeast Sae3-Mei5 complex still needs to be developed, the homologous complex from fission yeast, Swi5-Sfr1, has been shown to exhibit mediator function in Rad51 and Dmc1-mediated DNA strand exchange reactions (Haruta et al. 2006). For both DNA strand exchange proteins, Swi5-Sfr1 partially relieved the inhibition imposed by RPA binding to ssDNA. In contrast to *S. cerevisiae*, the fission yeast counterparts function in both vegetative and meiotic cells (Akamatsu et al. 2003, 2007; Ellermeier et al. 2004). Genetic analysis, in addition to the biochemical results, suggests that Swi5-Sfr1 is not specific for Dmc1 in fission yeast, and support a function for the complex in Rad51 filament formation (Akamatsu et al. 2003, 2007; Haruta et al. 2006). The mechanism by which Swi5-Sfr1 supports Rad51/Dmc1 filament formation on RPA-coated ssDNA remains to be determined.

2.3.6

Hop2-Mnd1: A Complex that Co-evolved with Dmc1

Hop2-Mnd1 form a conserved complex that appears to have co-evolved with the Dmc1 protein, as all organisms identified to have the Dmc1 protein also

contain the Hop2-Mnd1 complex, whereas organisms that lack Dmc1 also lack this complex (reviewed in Hunter 2007; Neale and Keeney 2006) (Fig. 4). This pattern may suggest an interaction between Hop2-Mnd1 and Dmc1 specific to a mechanism in meiosis, but biochemical results show that the mammalian and fission yeast complex can functionally interact with both Dmc1 and Rad51 (Chi et al. 2007; Enomoto et al. 2006; Petukhova et al. 2005; Ploquin et al. 2007). *hop2* and *mnd1* mutants in budding yeast, like *dmc1*, arrest at pachytene and sporulate only poorly (1.3% for *hop2* and 1.5% for *mnd1*, with no viable spores) (Leu et al. 1998; Tsubouchi and Roeder 2002). Unusual for *hop2* and *mnd1* among meiotic mutants is the failure of most chromosomes to properly pair with their homolog; instead, most chromosomes pair with non-homologous partners or are folded over as though synapsed with ectopic sites on the same chromatid. This anomalous pairing does not appear to be mediated at the level of DNA strand exchange intermediates, however, as joint molecules and COs are not detectable in the *mnd1* mutant (Gerton and DeRisi 2002). Hop2-Mnd1 may therefore function at or prior to DNA strand exchange to promote appropriate nucleoprotein filament interactions with a homologous target.

While the biochemical work is now focused on the Hop2-Mnd1 complex, Hop2 alone has been shown to catalyze ATP-independent D-loop formation. This activity is attenuated in the Hop2-Mnd1 complex (Petukhova et al. 2005; Pezza et al. 2006). The biological significance of this Hop2 activity remains unclear, because the genetic evidence suggests that Hop2-Mnd1 function as an obligatory heterodimer (Tsubouchi and Roeder 2002). The Hop2-Mnd1 complex stimulates the *in vitro* recombination activity of Dmc1 and Rad51, where tested (reviewed in Hunter 2007; Neale and Keeney 2006). Recent biochemical analysis identified two distinct mechanisms by which Hop2-Mnd1 enhance the function of Dmc1 and Rad51 (Chi et al. 2007; Pezza et al. 2007). First, Hop2-Mnd1 stabilized Rad51- or Dmc1-presynaptic filaments against disassembly. Second, Hop2-Mnd1 enhanced the ability of the Dmc1 or Rad51 presynaptic filament to capture duplex DNA in a homology-independent manner. The first function is akin to a mediator protein, and would predict an effect of *hop2/mnd1* mutants on Dmc1 or Rad51 focus formation. However, meiotic Rad51 and Dmc1 foci form normally in the *mnd1* and *hop2* mutants, and Mnd1 does not colocalize with Rad51 foci (Henry et al. 2006; Leu et al. 1998; Zierhut et al. 2004). Hence, there is no direct *in vivo* evidence that would support a role of Hop2-Mnd1 in presynapsis, although it is possible that the filaments formed in the absence of Hop2-Mnd1 are somehow different from those formed in wild-type cells. The second biochemical function of Hop2-Mnd1 in the homology search process is supported by the *in vivo* phenotype of the mutants, which display a complete homologous pairing defect. However, the absence of Mnd1 staining at recombination sites (Zierhut et al. 2004) is puzzling, and additional analysis will be needed to reconcile the biochemical, genetic, and cytological data.

2.3.7

The Breast Cancer Tumor Suppressor Protein BRCA2

The breast cancer tumor suppressor protein BRCA2 plays a role in HR in metazoans, plants, and at least one microbe (*Ustilago maydis*), but an obvious homolog is absent in budding and fission yeast (Fig. 4). BRCA2 is required for DNA damage-induced Rad51 focus formation (Tarsounas et al. 2003), and biochemical analysis of the *Ustilago* Brh2 protein and fragments of the human BRCA2 proteins have established its mediator function in targeting Rad51 filament formation on RPA-coated ssDNA to the ssDNA–dsDNA junction (Yang et al. 2002, 2005). Genetic evidence in *A. thaliana* implicates BRCA2 in meiosis, as silencing BRCA2 by RNAi caused meiotic defects and sterility (Siaud et al. 2004). BRCA2 was found to interact both with Rad51 and Dmc1 in *A. thaliana* and humans (Dray et al. 2006; Thorslund et al. 2007), suggesting that BRCA2 might play a similar mediator function for Dmc1 as it does for Rad51.

2.4

Formation of Heteroduplex DNA by Rad51 and Dmc1: Cofilaments or Asymmetry

Once assembled as functional filaments on ssDNA, both Rad51 and Dmc1 are homology search and DNA strand exchange proteins capable of DNA strand invasion. The efficiency and robustness of their *in vivo* reactions as well as the timing and routing (sister/homolog) are likely regulated by the various specific and common cofactors. Whereas only Rad51 functions in recombination in vegetative cells, both proteins are required for meiotic recombination and CO formation in most eukaryotes (Hunter 2007; Krogh and Symington 2004). How do Rad51 and Dmc1 perform their tasks during meiotic recombination? And why are two RecA homologs employed for meiosis?

Four models can be envisioned to explain how Dmc1 and Rad51 cooperate during meiotic recombination:

1. Rad51 and Dmc1 form mixed filaments
2. Cofilaments of Rad51 and Dmc1 patches on each resected DSB end
3. Rad51 and Dmc1 form asymmetric filaments, Rad51 on one end and Dmc1 on the second end of the DSB
4. Rad51 and Dmc1 consecutively load to form individual filaments on the same DNA at different times during meiosis (see Fig. 5)

The structural similarity of the presynaptic filaments formed by the RecA-like proteins (Egelman 2003) opens a possibility for the Rad51–Dmc1 cofilament scenario. Furthermore, in budding yeast, formation of Dmc1 foci is greatly reduced in *rad51* mutant cells (Shinohara et al. 1997a). Mouse Dmc1 and Rad51 interact and colocalize at recombination sites on meiotic chromosomes (Tarsounas et al. 1999). These observations seem compatible with a cofilament model. However, the fact that Dmc1 forms dHJs in *rad51* mutants argues

that Dmc1 can function in vivo in filaments not containing Rad51 (Schwacha and Kleckner 1997), consistent with the Dmc1 biochemistry. On the converse, Rad51 also evidently functions in the absence of Dmc1 during vegetative growth, and may even compensate for loss of Dmc1 function in meiosis under certain genetic conditions. In the *dmc1* background, a *hed1* mutation relieves Rad51 inhibition and partially rescues the *dmc1* spore viability defect, and overexpression of Rad51 similarly partially overcomes a *dmc1* defect (Tsubouchi and Roeder 2006). Most importantly, no biochemical evidence demonstrates the formation of mixed Rad51-Dmc1 filaments or cofilaments. An alternative to cofilaments is the formation of asymmetric filaments at the DSB site (Fig. 5, part B1). This was initially proposed on the basis of cytological data showing a close side-by-side localization of Rad51 and Dmc1 foci (Shinohara et al. 2000), but the mechanistic basis for such an asymmetry was difficult to reconcile with the apparent symmetric nature of the two ends of the DSB (see Fig. 2). However, analysis of the products of Spo11 cleavage revealed a surprising asymmetry in the oligonucleotides associated with Spo11 in vivo (Neale et al. 2005) (Fig. 3). This analysis showed that the Spo11 cleavage complex is associated with oligonucleotides of two discrete size ranges, either 10–15 nt or 24–40 nt. These oligonucleotide size classes could be the consequence of asymmetric processing of the two ends of a DSB. It was envisioned that the shorter oligo detaches to reveal a dsDNA–ssDNA junction for resection, whereas the longer oligo stays bound, creating a nick or gap for resection to commence (Neale et al. 2005). Such an asymmetry would result in two different substrates presented for end-processing at the DSB (nick versus 3'-overhang), resulting also in different substrates (tail versus gap) for Dmc1 or Rad51 filament assembly in DSBR or Rad52 loading for strand annealing during SDSA (Fig. 2). The two ends of a meiotic DSB are therefore not necessarily inherently symmetric, and a basis for potential asymmetric Rad51 and Dmc1 filament assembly is conceivable (Fig. 5, part C). Alternatively, the asymmetry of the meiotic DSB ends may provide a basis for temporally regulated, successive loading of Rad51 and Dmc1.

2.5

Roles of the Rad54 and Rdh54-Tid1 Motor Proteins in Presynapsis, Synapsis and Postsynapsis

Rad54 and Rdh54/Tid1 are closely related members of the Snf2-like family of dsDNA translocases with a partially overlapping function in meiosis. Biochemical experiments uncovered a surprising versatility of these enzymes, identifying potential functions at all three stages of HR: presynapsis, synapsis, and postsynapsis (Figs. 2, 4, 6), which are discussed in this section. In budding yeast, 30% of *rad54*- Δ mutants form spores, of which 53% are viable (compared to 88% spore formation in wild-type with 98% viability). Similarly, 10–44% of *rdh54*- Δ /*tid1*- Δ mutants form spores, of which 64–82% are

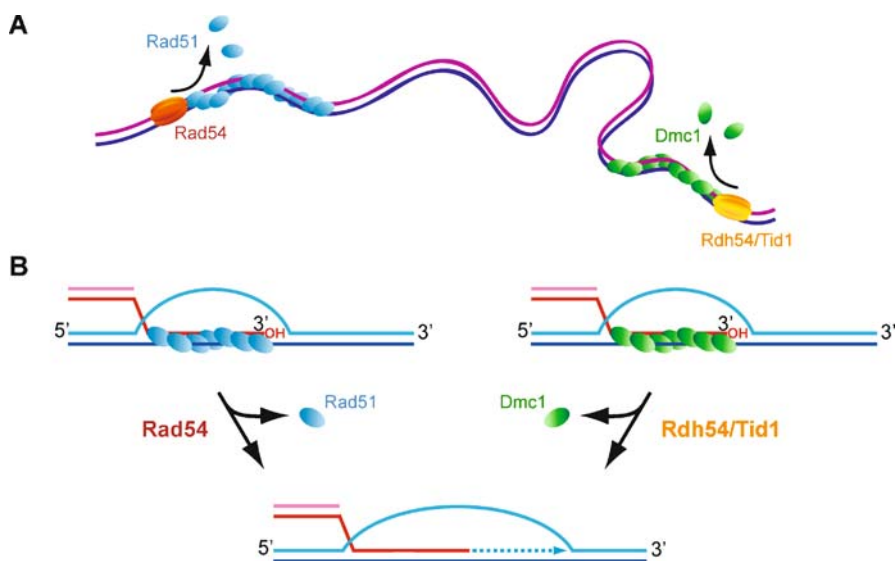


Fig. 6 Rad54 and Rdh54-Tid1: removal of Rad51 from dsDNA and DNA polymerase extension from the 3' end of heteroduplex DNA. Rad51 and Dmc1 bind readily to dsDNA, unlike their bacterial counterpart RecA. Rad54 and Rdh54/Tid1 may function to dissociate Rad51-dsDNA complexes or Dmc1-dsDNA complexes, in at least two contexts: **A** non-specific binding to dsDNA ("dead-end" complexes), and **B** turnover from the heteroduplex dsDNA product of DNA strand exchange to allow access of DNA polymerase to the invading 3' end. Not all activities depicted here have been experimentally demonstrated, but are inferred from *in vivo* and *in vitro* results with these proteins (for details see text)

viable. The meiotic defect in the *rad54 rdh54* double mutant, however, rivals that of the *rad51 dmc1* double mutant, virtually eliminating HR. Like the *rad51 dmc1* double mutant, the *rad54 rdh54/tid1* double mutant accumulates hyper-resected DSBs and produces few recombinants; consequently, spore formation is reduced to 0.5%, of which a mere 1.6% are viable (Klein 1997; Shinohara et al. 1997b). The potential functional overlap could be a consequence of the shared interaction of Rad51 with both Rdh54/Tid1 and Rad54, whereas Dmc1 appears to interact only with Rdh54/Tid1 (Clever et al. 1997; Dresser et al. 1997; Jiang et al. 1996).

Rad54 and Rdh54/Tid1 display exceedingly similar biochemical characteristics, although not all experiments have been performed with both enzymes (for review Heyer et al. 2006; Tan et al. 2003). Both proteins are dsDNA-dependent motor proteins, and single molecule studies determined that Rad54 translocates at ~ 300 bp/s in a processive manner on average for 11.5 kb, whereas Rdh54/Tid1 translocates at ~ 100 bp/s for an average of 10 kb (Amitani et al. 2006; Nimonkar et al. 2007; Prasad et al. 2007). Comparison of the phenotypes of ATPase-deficient Rad54 mutants with those

caused by the gene deletion demonstrates that the ATPase activity is critical for in vivo function (Clever et al. 1999; Petukhova et al. 1999b). Using the same Walker A box ATPase mutation, biochemical and in vivo studies identified ATP-dependent and ATP-independent functions of Rad54 (and possibly by implication Rdh54/Tid1). Together these results show that Rad54 serves motor-dependent and motor-independent roles in HR (see below). Many of these roles may relate directly to an interaction with Rad51, at the level of nucleoprotein filament dynamics (Rad51 stability on ssDNA), DNA strand exchange, heteroduplex DNA extension (branch migration), and DNA repair synthesis from the heteroduplex product of DNA strand exchange (Rad51 dissociation from duplex DNA) (Heyer et al. 2006).

During presynapsis, Rad54 stabilizes Rad51-ssDNA filaments in an ATP-independent fashion (Mazin et al. 2003) (Fig. 4). ChIP experiments provided evidence for the in vivo significance of this stabilization function, demonstrating enhanced association of Rad51 with the proximal end of the resected DNA strand in a strain expressing an ATPase-deficient Rad54 mutant (Rad54-K341R), but not in a *rad54*- Δ strain (Wolner and Peterson 2005). Association of Rad54 with the presynaptic filament effectively targets Rad54 to the pairing site, where it can exert its motor function on dsDNA (Mazin et al. 2000a; Solinger et al. 2001; Van Komen et al. 2000). A similar function has not yet been directly demonstrated for Rdh54/Tid1.

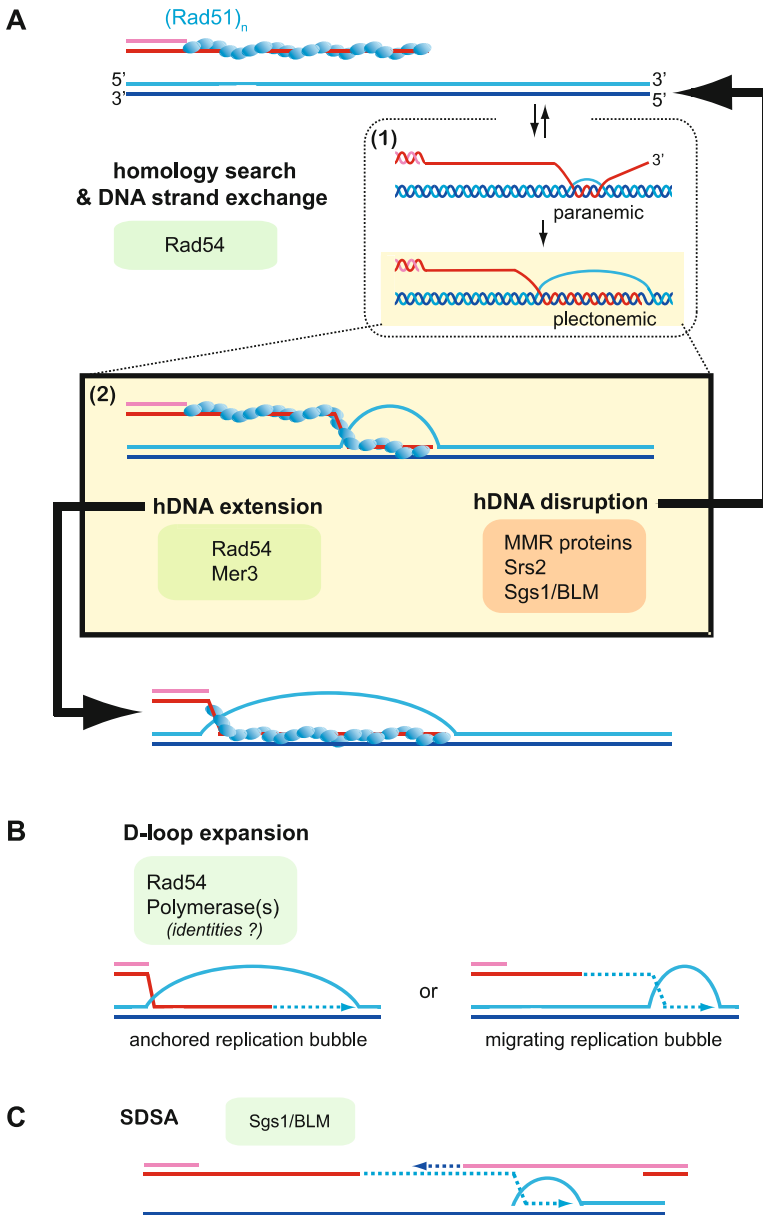
Synapsis entails homology search and DNA strand invasion (Figs. 2, 7). Rad54 stimulates the Rad51-mediated DNA strand exchange reaction in vitro (circular ssDNA invading linear dsDNA; for a review of biochemical recombination assays see Heyer 2007) and Rad51-mediated D-loop formation (linear ssDNA invading supercoiled dsDNA) (Mazin et al. 2000b; Petukhova et al. 1998). The mechanism of this stimulation has not been elucidated yet, but requires the motor function of Rad54, since it depends on Rad54 ATPase activity (for review Heyer et al. 2006; Tan et al. 2003). In vivo, Rad54 is not absolutely required to target the Rad51 filament to the pairing site as monitored by ChIP experiments (Sugawara et al. 2003), suggesting that homology search can proceed in a Rad54-independent fashion. However, it is nevertheless possible that Rad54 contributes to this process in vivo (Wolner et al. 2003). The ChIP experiments (Sugawara et al. 2003; Wolner et al. 2003) cannot determine whether DNA strand invasion depends on Rad54, as they do not determine the structure of the DNA intermediate bound by the proteins. Rdh54/Tid1 also stimulates Rad51-mediated D-loop formation in vitro (Petukhova et al. 2000), but this activity has not yet been demonstrated with Dmcl.

A distinct role of Rad54 after DNA strand invasion (postsynapsis) is indicated by the specific stimulation of the Rad54 ATPase activity at the termini of Rad51-dsDNA filaments (Kiiianitsa et al. 2002, 2006; Li et al. 2007). Rad51-dsDNA complexes may represent dead-end complexes caused by the binding of Rad51 to chromosomal (duplex) DNA, or they may constitute the product complex of DNA strand invasion, when Rad51 is bound to the heteroduplex

DNA (Fig. 6). Snf2-like proteins remodel a diverse array of protein-duplex DNA complexes, including nucleosomes and transcription complexes (Pazin and Kadonaga 1997). Rad54 remodels the Rad51-dsDNA filament, leading to the dissociation of Rad51 from duplex DNA (Solinger et al. 2002). The low dsDNA ATPase activity of Rad51 (over 100-fold lower than RecA) and the stability of the Rad51-dsDNA complexes even under conditions of ATP hydrolysis (Li et al. 2007; Sung 1994) suggest that Rad54 acts as a turnover factor for Rad51 (Solinger et al. 2002). This activity is likely critical to allow DNA polymerases access to the invading 3'-end, since catalytic turnover of RecA is required for this step (Xu and Marians 2002). This notion is consistent with the finding that meiotic Rad51 foci do not colocalize with recombination-dependent DNA synthesis (Terasawa et al. 2007), suggesting that Rad51 needs to dissociate from the hDNA before DNA synthesis can extend the D-loop. In vivo experiments demonstrate that in Rad54-deficient cells no DNA synthesis takes place at the pairing site (Sugawara et al. 2003), although the lack of DNA synthesis could be a downstream consequence of a role of Rad54 in forming the DNA pairing intermediate (D-loop) required for extension. Rdh54-Tid1 has also been demonstrated to dissociate Rad51-duplex DNA filaments (Chi et al. 2006). This activity has not yet been demonstrated for Dmc1-dsDNA complexes, although the biochemical properties of Dmc1 (low dsDNA-dependent ATPase, dsDNA binding) suggest that Dmc1 might also form dead-end complexes on duplex DNA and remain stuck on the heteroduplex DNA after DNA strand exchange. Indeed, elegant in vivo experiments demonstrated that in *tid1/rdh54* mutants Dmc1 accumulates at non-DSB sites, suggesting a function of Rdh54/Tid1 in dissociating dead-end complexes of Dmc1 on duplex DNA during meiosis (Holzen et al. 2006).

A second potential role of Rad54 in postsynapsis is in branch migration (Fig. 7), and Rad54 motor activity was shown to enhance branch migration in Rad51-mediated in vitro recombination reactions (Bugreev et al. 2006; Solinger and Heyer 2001). Stimulation of branch migration was observed during Rad51-mediated DNA strand exchange and on protein-free junctions. The in vivo significance of these biochemical data remains uncertain. Overexpression of wild-type Rad54 protein leads to a reduction in conversion tract length, whereas overexpression of an ATP-deficient mutant Rad54 protein increased conversion tract length (Kim et al. 2002). These results appear inconsistent with a role for Rad54 in driving branch migration (defined as heteroduplex extension), which would be expected to lead to an increase in conversion tract length. However, this capacity to move DNA junctions has also been proposed to lead to the disruption of recombination intermediates (see below) enabling second end capture (Bugreev et al. 2007a), which would curtail conversion tract length. Similar experiments have not yet been conducted with Rdh54/Tid1 protein.

As members of the Snf2-like protein family (Flaus and Owen-Hughes 2004), Rad54 and Rdh54/Tid1 are related to prominent chromatin remod-



eling factors but also to other factors that have non-chromatin remodeling targets like Mot1, which dissociates TBP from the TATA-box (Sprouse et al. 2006). The Snf2-like chromatin remodeling factors function as a single sub-unit in large hetero-multimeric assemblies, whereas Rad54 and Rdh54/Tid1 form homo-multimeric assemblies, possibly hexameric or double-hexameric

- ◀ **Fig. 7** Joint molecule dissociation versus maturation by hDNA extension and D-loop expansion. **A** Homology search and DNA strand invasion by the Rad51 (or Dmc1, not shown) filament leads to the D-loop intermediate. Strand invasion by an incomplete filament or DNA strand invasion initiating internally (not at end) generates a paranemic joint, where the invading strand is not fully intertwined with the template strand and pairing is protein-mediated. (See *box 1* for a representation, although the true nature of a paranemic interaction is uncertain. The Rad51 protein is not shown for simplicity.) Paranemic joints are unstable and may revert, or the pairing is extended to the end, allowing formation of a plectonemic joint with full strand intertwining in which heteroduplex base-pairing is sufficient for stability of the DNA strand exchange product (as explicitly drawn in *box 1*). Rad51 filament nucleation at the dsDNA–ssDNA junction would increase the probability of interstitial pairing resulting in paranemic joints. The D-loop drawn in *box 2* is a plectonemic joint, but for simplicity strand intertwining is not drawn. The D-loop may be disrupted in processes averting recombination or during SDSA (after DNA synthesis, see *C*) involving MMR proteins, Srs2, and RecQ-like DNA helicases (see text for details). Alternatively, the D-loop can be enlarged by hDNA extension, and the Rad54 motor protein as well as the Mer3 DNA helicase have been implicated in this step. Mer3 plays a key role in CO formation through CO pathway 1 (see text). The extended D-loop is possibly the metastable intermediate SEI that is specific for CO pathway 1 (Hunter and Kleckner 2001). **B** The initial D-loop may also be expanded by DNA synthesis as an anchored bubble (*left*) or may become a migrating bubble (*right*), where its size remains unchanged (Formosa and Alberts 1986). **C** SDSA is effectively D-loop expansion coupled to regulated hDNA and D-loop disruption. Presumably, D-loop disruption is initiated after homology quality check has sanctioned DNA synthesis. hDNA extension and D-loop expansion are not necessarily stable end-points. DNA strand invasion and SDSA may be dynamic sampling states, where joint molecule formation and disruption occur iteratively, explaining the identification of genetic information obtained from multiple donor sites (Symington and Heyer 2006)

rings as suggested by the processivity of their translocation (Amitani et al. 2006; Flaus and Owen-Hughes 2004; Kiianitsa et al. 2006; Nimonkar et al. 2007; Prasad et al. 2007). Rad54 slides mono-nucleosomes in vitro (Alexeev et al. 2003; Jaskelioff et al. 2003) and enables DNA strand invasion on nucleosomal templates (Alexiadis and Kadonaga 2003; Zhang et al. 2007). Whether Rad54 is required for chromatin remodeling in vivo remains to be demonstrated. Direct analysis of a positioned nucleosome at the recombination target during mating-type switching did not reveal a function of Rad54 in chromatin remodeling during HR (Wolner and Peterson 2005). The activity of Rdh54/Tid1 on nucleosomal substrates remains to be tested.

As for Rad51 and Dmc1, Rad54 and Rdh54/Tid1 share key biochemical properties. The nature of the specialization of Dmc1 and Rdh54/Tid1 for meiotic recombination therefore remains to be satisfactorily explained, as does the likely regulation of Rad51 and Rad54 in association with their meiotic paralogs. While Rad54 is as essential for recombination in mitotic cells as Rad51, in meiosis Rad54 is more involved in sister chromatid repair than interhomolog recombination (Arbel et al. 1999). Rdh54, in contrast, plays little role in mitotic recombination, but is more critical in meiosis (Klein 1997; Shinohara et al.

1997b). The important role of Rdh54 in interhomolog recombination is likely mediated by its interaction with Dmc1 (Dresser et al. 1997).

2.6

DNA Synthesis: Involvement of the PCNA/RFC-Dependent Pol δ and Possibly Pol λ

Whereas factors associated with Rad51 and Dmc1 filament assembly and DNA strand exchange have been identified, little is known about the proteins and the mechanisms involved in extending the invading 3'-end in the D-loop (Fig. 7, part B). This makes it difficult to anticipate variations unique to meiotic DNA repair synthesis, as so little is known about factors that accomplish DNA repair synthesis during recombination in vegetative cells. The multitude of nuclear DNA polymerases (Rattray and Strathern 2003) and the likely involvement of polymerase processivity factors such as PCNA and RFC provides for significant complexity at this step. Genetic evidence implicates DNA polymerase δ in meiotic DNA repair synthesis, as a hypomorphic allele of the catalytic subunit encoded by the *POL3* gene displays reduced meiotic conversion and lower CO frequency (Maloisel et al. 2004). Since Pol δ is a PCNA-dependent DNA polymerase, it is expected that the processivity clamp and the RFC clamp loader are required as well. Estimates for the extent of resection (on average 500 nucleotides per break end) (Sun et al. 1991) provide a minimum for the new DNA synthesis required. This estimate also supports the involvement of a processive, PCNA-dependent polymerase.

Another DNA polymerase with a possible role in meiotic recombination is Pol λ , a nonessential, Pol β -like enzyme encoded by the *POL4* gene of *S. cerevisiae* (Shimizu et al. 1993). Mutants in *POL4* display a fivefold elevated frequency of intragenic recombination and a several-fold increase in the steady state level of Spo11-induced meiotic DSBs (Leem et al. 1994). This suggests a function in recombination downstream of DSB formation. The *POL4* locus expresses a meiosis-specific transcript, in addition to a constitutive transcript (Leem et al. 1994), and also the protein level appears to be increased in meiosis (Shimizu et al. 1993). Nevertheless, spore viability is normal in *pol4* mutants. The biochemical characteristics of Pol λ purified from vegetative (mitotic) and meiotic cells were reported to be nearly identical (Shimizu et al. 1993). Pol β typically inserts one to few nucleotides, for example during base excision repair (Lindahl and Wood 1999), but processivity has not been tested with the budding yeast Pol λ enzyme.

Recent evidence suggested the involvement of DNA polymerase η in D-loop extension reconstituted in vitro with human proteins and in certain recombination events in chicken DT40 cells (Kawamoto et al. 2005; McIlwraith et al. 2005). Pol η has a well-established role in bypassing UV photoproducts and there is presently no genetic or biochemical evidence linking Pol η in *S. cerevisiae* to HR. Considering functional overlap with other polymerases, however, such a role cannot be ruled out presently.

2.7

D-Loop Dissolution and Strand Annealing in SDSA

In addition to being the DNA structure in which DNA repair synthesis initially occurs, the D-loop is in fact a key intermediate at which meiotic “choices” for CO and NCO pathways are thought to be made (Figs. 2, 7). Most DSBs generated by Spo11 are processed to NCO, likely involving an SDSA mechanism. Some DSBs (estimated to be about one third) mature along a CO-designated pathway, primarily envisioned as a variant of the DSBR model. The recombination pathways as depicted in Fig. 2 bifurcate at the D-loop intermediate to promote either (1) second end capture and dHJ formation (DSBR pathway; see below) or (2) disengagement of the interaction by D-loop dissolution and annealing of the newly synthesized strand to the second end (SDSA pathway). It has been proposed that the SEI intermediate identified in physical assays is a D-loop with extended heteroduplex stabilized by branch migration, but not necessarily expanded by DNA repair synthesis from the invading end. The SEI represents an early CO-dedicated intermediate; as dHJs are also CO-dedicated, SEIs presumably develop along the dHJ pathway (Borner et al. 2004; Hunter and Kleckner 2001) (reviewed in Hunter 2007). The consequence of pathway divergence at the level of the D-loop would be that the D-loops in the DSBR and SDSA pathways are somehow distinct, possibly with different physical properties. What determines these early distinctions is currently unknown.

Dissolution of D-loops is the distinguishing reaction of the SDSA pathway (Figs. 2, 7). From biochemical analysis, the primary candidates for this activity are the RecQ-like DNA helicases. The biochemistry with the budding yeast RecQ helicase, Sgs1, is underdeveloped due to challenges purifying the enzyme, but the human BLM and WRN DNA helicases were shown to dissolve D-loop substrates (Bugreev et al. 2007b; Orren et al. 2002; van Brabant et al. 2000). In the case of BLM, D-loops appear to be the preferred substrate (Bachrati et al. 2006). The involvement of BLM in D-loop dissolution has also been proposed on the basis of genetic results in *Drosophila* (Adams et al. 2003; McVey et al. 2004a,b). Genetic evidence for such a function of Sgs1 in budding yeast is lacking, but an analysis of mitotic recombination events implicated the unrelated Srs2 helicase in D-loop dissolution during SDSA (Ira et al. 2003). A possible functional overlap of both types of DNA helicase is indicated by the observation that overexpression of Sgs1 can rescue the MMS- and HU-sensitivity of an *srs2* mutant (Mankouri et al. 2002). However, it is unclear whether this can be interpreted as functional equivalence or compensation of a pathological situation. No Srs2 homolog has been identified in metazoans yet, opening the possibility that one of the RecQ-like helicases has usurped the D-loop dissolution function. The biochemical analysis of Srs2 identified a novel mechanism of anti-recombination distinct from D-loop dissolution: disassembly of the Rad51 presynaptic filament (Krejci et al. 2003;

Veaute et al. 2003). This mode of action is consistent with, and in fact was suggested by, the genetic analysis of semidominant *RAD51* suppressors of the *srs2* phenotype (Aboussekhra et al. 1992). Also, the mammalian BLM and RECQL5 DNA helicases were demonstrated to dissociate Rad51 from ssDNA (Bugreev et al. 2007b; Hu et al. 2007). The biochemical experiments (Krejci et al. 2003; Veaute et al. 2003) also examined the possibility that Srs2 disrupts D-loops, but Srs2 was unable to dissociate D-loops once formed. This suggests that Srs2 might need another cofactor or different reaction conditions to catalyze D-loop dissolution. In sum, the genetic evidence favors Srs2 as the helicase that dissolves D-loops in budding yeast, but the biochemical support for this proposal still needs to be forthcoming.

Like the RecQ helicases, human Rad54 was also reported to dissociate D-loops in vitro (Bugreev et al. 2007a). A role of Rad54 in D-loop dissociation appears counterintuitive, because of its stimulation of Rad51-mediated D-loop formation in the yeast and human systems (Mazin et al. 2000b; Sigurdsson et al. 2002). However, when the reaction is staged in vitro such that Rad54 is added after D-loop formation, Rad54 dissociates deproteinized D-loops in an ATPase-dependent fashion (Bugreev et al. 2007a). The in vivo significance of these biochemical results is uncertain. Such an activity would predict an anti-CO function for Rad54, which has not been identified in budding yeast (Krogh and Symington 2004; Paques and Haber 1999). Moreover, extensive in vivo analysis by ChIP and PCR assays has identified an absolute defect in *rad54* mutants to extend (or make) D-loops (see discussion above) (Sugawara et al. 2003; Wolner et al. 2003). Since D-loop extension precedes D-loop dissolution in the SDSA model, D-loop dissociation cannot be the first rate-limiting Rad54 function, at least in budding yeast. It will be difficult to genetically test whether Rad54 might have a role at this step, as it will require specific mutants that can separate the earlier function (D-loop formation/extension) from the proposed later function (D-loop dissociation).

D-loop dissolution during SDSA is likely coordinated with the release of the newly synthesized DNA from its template, to allow annealing of the disengaged, extended single strand with the second resected DSB end (Fig. 7, part B). DNA strand annealing between these ssDNA species involves RPA and Rad52, possibly involving a contribution by Rad59 protein. A role for Rad52 in annealing complementary ssDNA in fact is supported for both SDSA and DSBR, suggesting that strand annealing is a common feature of both NCO and CO meiotic repair pathways (Lao et al. 2007; Sugiyama et al. 2006). It follows that a primary difference in SDSA and DSBR pertains not to the fate of the second end, but to the fate of the first invading end (see Sect. 2.8). In the nuclear context, newly generated ssDNA can be thought of as an ssDNA-RPA complex, because of the high affinity of RPA binding to ssDNA (Wold 1997). RPA binding provides an effective barrier against spontaneous reannealing of ssDNA. Rad52, like its bacterial counterpart RecO, is special in its ability

to reanneal ssDNA complexed to the cognate ssDNA binding protein, RPA in eukaryotes (Kantake et al. 2002; Mortensen et al. 1996; Sugiyama et al. 1998, 2006). This activity is also key for SSA, a DSB repair pathway that is not further discussed here (see Paques and Haber 1999). The unique role of Rad52 in annealing and its mediator activity in Rad51 filament formation (see above) provide the mechanistic explanation for why *rad52* mutants display the most extreme recombination defect in budding yeast. The *RAD59* gene was identified in a screen for recombination factors acting in a Rad51-independent fashion, and is likely specific for the SSA pathway (Bai and Symington 1996). Rad59 is a Rad52 paralog, sharing Rad52's N-terminal DNA binding domain but lacking the C-terminal Rad51 interaction domain. The protein functions in conjunction with Rad52, as indicated by the observed suppression of *rad59* mutants by Rad52 overexpression and their mutual physical interaction (Bai and Symington 1996; Davis and Symington 2001, 2003). Like Rad52, Rad59 can reanneal DNA (Davis and Symington 2001; Petukhova et al. 1999a), but not in the presence of RPA (Wu et al. 2006b), suggesting that Rad59 is unlikely to function in a Rad52-independent fashion during strand annealing in vivo. The specific mechanistic contribution of Rad59 to Rad52-mediated annealing remains to be determined, and it is unclear whether Rad59 becomes part of the Rad52 ring or how it associates with the Rad52 ring structure.

2.8

Second End Capture in DSBR

Although SDSA is thought to process the majority of DSBs induced during meiosis, it has no contribution to CO or to chiasmata (Fig. 2). Its NCO outcome does, however, contribute to meiotic homolog segregation because it achieves the physical repair of most DSBs and effectively limits the number of COs per chromosome interval. Too many COs may be as detrimental as too few COs. An excess of COs may interfere with homolog disjunction during meiosis I by making physical segregation too difficult; too closely spaced COs may leave insufficient sister cohesion in place to stabilize the chiasma. The primary CO pathway in budding yeast (and by implication of the genetic requirements in *C. elegans* and mammals) is defined by dHJs. dHJs develop from a minority of the meiotic DSBs and require engagement of the two DSB ends, which can occur by at least two different mechanisms. Both ends could invade independently of each other, or the second end could be captured by the displaced strand of the D-loop. As in the SDSA pathway, the second end capture model implies an asymmetry between the invading end (presynaptic filament) and the second end (RPA-Rad52) (see Sect. 2.7). The displaced strand of the D-loop is likely bound by RPA (Eggleter et al. 2002), and capture of the second end involves Rad52 (Lao et al. 2007; Sugiyama et al. 2006) and possibly Rad59. The reaction is similar but not identical to

the reannealing step in SDSA. The basic biochemistry of these proteins and the reannealing reactions they catalyze were summarized above. It is unclear whether the specific topology in the D-loop provides constraints in the annealing reaction that require additional factors (Rad59?). Physical evidence from budding yeast is consistent with a second end capture mechanism promoted by Rad52: analysis of a *rad52-327* separation of function allele that is deficient in Rad51 mediator function but proficient in annealing of complementary ssDNA showed that Rad52 promotes the transition from SEIs to dHJs (Lao et al. 2007). Capture of the second end by an annealing mechanism is not easily matched with the proposal that Dmc1 and Rad51 form distinct filaments each on one end of the DSB (discussed under Sect. 2.4). A possible solution is that these processes occur sequentially, with initial Rad51 end invasion into the sister, followed by dissolution of the joint, and subsequent second-end capture to form an interhomolog dHJ (see Hunter 2007; Oh et al. 2007).

Although second end capture is viewed as an intermediate to the formation of dHJs and ultimately COs during meiotic HR (Mazina et al. 2004; Sugiyama et al. 2006), this notion has been challenged on the basis of biochemical experiments with human Rad54 protein (Bugreev et al. 2007a). Elegantly designed biochemical experiments reconstituted major steps of DSB repair in vitro. These included Rad51-mediated strand invasion (tailed DNA with supercoiled dsDNA) and second end capture mediated by Rad52, resulting in double-D-loop structures that are equivalent to dHJs prior to ligation of the nicked strands (see Fig. 2 intermediate 5a; Fig. 11) (Bugreev et al. 2007a). Staged addition of Rad54 to these intermediates resulted in their dissolution, leading to the proposal that second end capture is not necessarily a CO-dedicated intermediate in HR (Bugreev et al. 2007a). The biological significance of this biochemical activity of Rad54 is uncertain, since the predicted anti-CO function for Rad54 has not been identified (Krogh and Symington 2004; Paques and Haber 1999). Moreover, a sole function of Rad54 at this step is contradicted by in vivo data, showing a Rad54 requirement for strand invasion and/or D-loop extension (see above) (Sugawara et al. 2003; Wolner et al. 2003).

2.9

Branch Migration in D-Loops and Double Holliday Junctions

Branch migration can occur at structural intermediates such as D-loops, single HJs, or dHJs, and may promote the interconversion of 3' to 5' flap structures. In D-loops, active and directed branch migration by an activity dedicated to the movement of a specific joint molecule species either enlarges or dissolves the structure (heteroduplex extension or D-loop disruption; Fig. 7) and may have key consequences for the stability of a DNA strand exchange product and its ultimate maturation along a CO- or NCO-designated path-

way. An alternative application for branch migration entails migration of the D-loop bubble in conjunction with DNA synthesis (D-loop expansion), as suggested for T4 recombination (Formosa and Alberts 1986). In either case, branch migration refers to the transplacement of a joint molecule relative to the initial position of DNA strand exchange, and may occur as an active process of joint molecule migration, or as an indirect consequence of DNA synthesis within the D-loop. In dHJs, branch migration may either coordinately move the double junction or alter the distance between the component junctions (for further discussion, see Hunter 2007). Although there may be no change in net base pairing, branch migration is likely driven by specific motor proteins and may also require topoisomerase contributions to relieve superhelical strain. The paradigm for a branch migration protein is the bacterial RuvAB complex, which enforces a square planar configuration on a HJ and moves the junction by pumping DNA through two appropriately positioned hexameric rings that are coordinated by a RuvA tetramer (West 1997). This branch migration enables the sampling of DNA sequences for preferred incision sites cleaved by the RuvAB-associated HJ resolvase, RuvC. Eukaryotes do not have obvious sequence homologs of the RuvABC complex, and whether a similar branch migration-resolution mechanism applies to eukaryotic recombination is not known. At any rate, HJ migration to extend hDNA may not be needed to explain heteroduplex tract lengths in meiosis. DSB resection extends on average 500 nucleotides from each break end, a span that is close to estimates of the average gene conversion tract lengths associated with CO (Paques and Haber 1999; Sun et al. 1991). Resection might therefore be sufficient to define the extent of the hDNA. Here, we discuss the DNA helicase Mer3 that is able to promote branch migration of D-loops. In addition, the function of additional pro-CO factors, Msh4-Msh5 and Mlh1-Mlh3, and their relationship to dHJs will be considered.

2.9.1

The Meiosis-Specific Mer3 DNA Helicase

Mer3 is a meiosis-specific DexH-box-type 3'-5' DNA helicase that functions in the SEI-dHJ CO pathway (Figs. 2, 7), together with Msh4-Msh5, Mlh1-Mlh3 and Exo1 (Borner et al. 2004; Hunter 2007; Nakagawa and Kolodner 2002a; Nakagawa and Ogawa 1999). *mer3-Δ* mutants have reduced sporulation (~24% relative to 61% in wild-type) with reduced viability (20–40% relative to 95% in wild-type), probably explained by the reduction in COs in the mutant (Nakagawa and Ogawa 1999). In five intervals studied on two different chromosomes, COs are reduced on average 2.4-fold relative to wild-type and result in an increased incidence of two- and zero-spore tetrads, consistent with meiosis I non-disjunction. Moreover, the COs that remain in the *mer3-Δ* mutant are distributed randomly, indicating an absence of crossover interference that suggests that CO pathway 1 in budding yeast de-

depends on *MER3* (Nakagawa and Ogawa 1999). Interestingly, *mer3* alleles with mutations in the Walker A-type ATPase boxes reduce CO and interference in some, but not all, of the loci tested (Nakagawa and Kolodner 2002a).

Biochemical analysis of purified Mer3 protein demonstrated 3'-5' DNA helicase activity, preferentially unwinding substrates with a 3' tail but also HJs assembled from oligonucleotides (Nakagawa et al. 2001; Nakagawa and Kolodner 2002a,b). HJ unwinding in vitro is challenged, however, by the addition of NaCl (to 150 mM) in the reaction, which does not affect the activity of Mer3 on other DNA substrates. The salt effect may reflect a stabilization of blunt ends and suggests HJ disruption may not represent a normal biochemical property of the protein (Nakagawa and Kolodner 2002b). Rather the physiological target of Mer3 is likely a D-loop as discussed below.

In Rad51-catalyzed DNA strand exchange, Mer3 stimulates hDNA extension when strand exchange is initiated in a 3' to 5' direction with reference to the invading strand (equivalent to enlarging the D-loop after 3' end invasion; see Fig. 7), whereas it inhibits DNA strand exchange in the opposite direction (Mazina et al. 2004). Rad51-catalyzed DNA strand exchange has been reported to proceed in either a 3' to 5' or 5' to 3' direction in vitro (Namsaraev and Berg 1998). However, it is widely believed that the 3' end invades to prime DNA synthesis and that filament formation is initiated at the dsDNA-ssDNA junction, biasing filament growth 5' to 3' and therefore DNA strand exchange 3' to 5' (Fig. 2). As a consequence, Mer3 is envisioned to enlarge the D-loop by translocating on the displaced strand and to counteract non-productive invasion of the 5' end (Mazina et al. 2004). Kinetic analysis showed that Mer3 did not enhance joint formation, but specifically stimulated nicked circle formation, an indicator of completed DNA strand exchange. This biochemical function to stabilize nascent DNA strand invasions is consistent with a pro-CO role of Mer3 in generating dHJ intermediates (Borner et al. 2004; Hunter 2007). Although tested in vitro with Rad51, the role of Mer3 in meiotic heteroduplex extension may also (or more importantly) extend to branch migration of D-loops catalyzed by Dmc1. This has not been tested yet in vitro for Dmc1 or mixed Dmc1-Rad51 reactions, but the requirement for Dmc1 in homolog-directed SEIs in vivo indicates that Dmc1-associated heteroduplex tracts are the targets for extension along the dHJ-associated CO pathway.

2.9.2

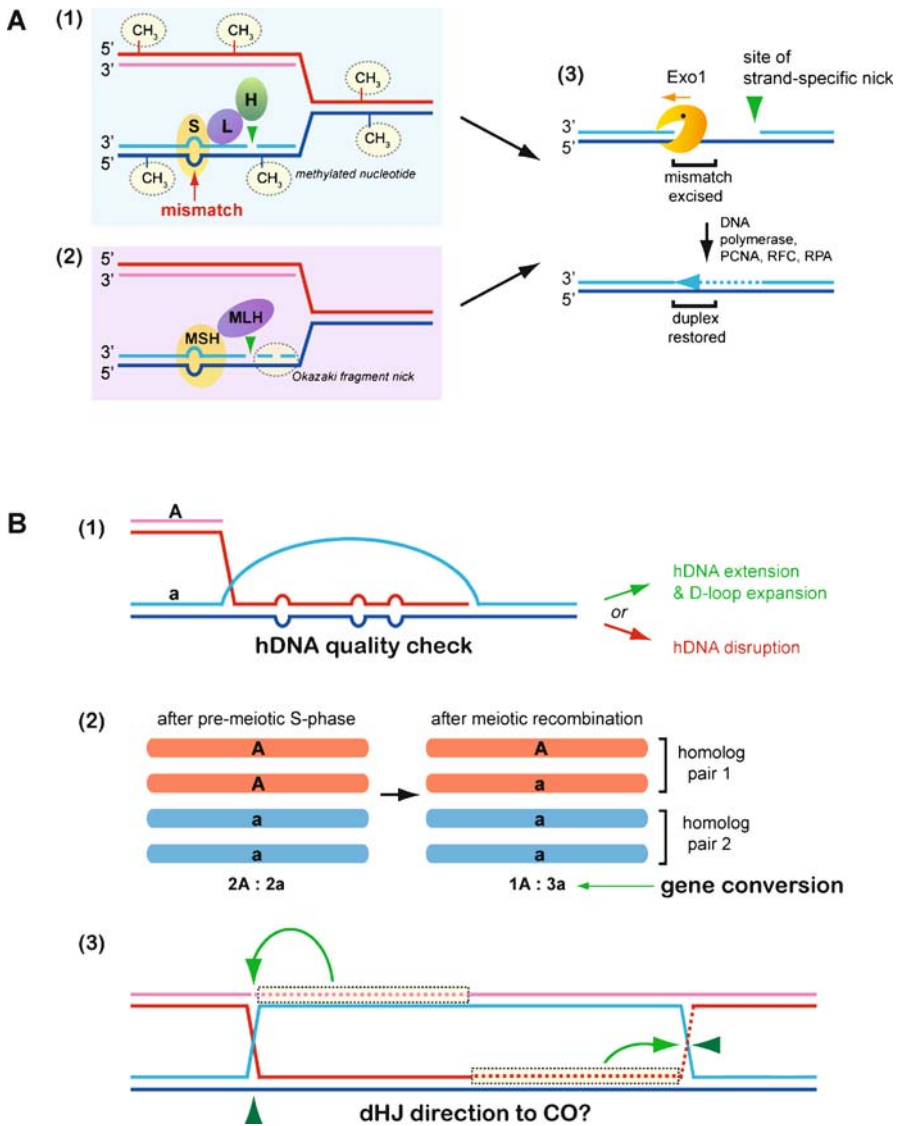
The Meiosis-Specific MutS Homolog Complex Msh4-Msh5 and dHJ Stabilization

Eukaryotic homologs to the bacterial MutS and MutL proteins function in heterodimeric assemblies (in *S. cerevisiae* Msh2-Msh3, Msh2-Msh6, Msh4-Msh5 and Mlh1-Mhl2, Mlh1-Pms1, Mhl1-Mlh3, respectively; see Sect. 2.10, Table 1). Besides their characteristic function in postreplicative MMR, these

complexes, with the exception of Msh4-Msh5, function also in the processing of mismatches in heteroduplex DNA formed during meiotic recombination (Hoffmann and Borts 2004; Schofield and Hsieh 2003) (see below and Fig. 8). Msh4-Msh5 has a unique role to promote meiotic COs by the dHJ pathway (Figs. 2, 9). Similar to MMR, the Msh4-Msh5 heterodimer functions in conjunction with a MutL heterodimer, namely Mlh1-Mlh3, in the major CO pathway in budding yeast involving the SEI-dHJ intermediates (Hoffmann and Borts 2004).

Msh4 was identified in a screen for meiosis-specific transcripts; its budding yeast mutants are normal for mismatch correction and gene conversion, but show 30–50% reduction in meiotic COs and consequently meiosis I non-disjunction, resulting in reduced spore viability (Ross-Macdonald and Roeder 1994). Msh5 was identified in a genetic screen for mutants deficient in recombination between, but not within, homologs (Hollingsworth et al. 1995). Like other Msh heterodimers, Msh4-Msh5 responds to target structures in DNA by exchanging ADP for ATP, a biochemical switch that induces clamp-like binding to the DNA (Schofield and Hsieh 2003; Snowden et al. 2004). Once loaded on DNA, the ATP-charged complex diffuses or slides along the duplex away from the initial site of binding (Fig. 9). In the case of MutS and its orthologs, iterative loading and sliding events promote the accumulation of MutS complexes in the vicinity of the single mismatch, amplifying the signal to downstream effectors and probably contributing to a local state that defines the extent of subsequent excision and resynthesis (Schofield and Hsieh 2003). Unlike Msh2-Msh3 or Msh2-Msh6, however, Msh4-Msh5 shows little binding to a mismatch *in vitro*. Instead, its target DNA structural features are found in joints *between* DNA duplexes (D-loops and HJs) and not simply irregularities within one duplex (Snowden et al. 2004). This sets Msh4-Msh5 apart from its vegetative paralogs, such as Msh2-Msh6, which bind a more degenerate range of junctions that includes splayed Y and duplex DNA with a G/T mismatch. Indeed, the Msh4-Msh5 preference for binding recombination-associated joint molecules is probably a direct reflection of its *in vivo* contribution to CO.

Based on these biochemical properties, Msh4-Msh5 is proposed to promote COs by the key property of encircling both homolog duplexes (Snowden et al. 2004) (Fig. 9). Topological tethering of homologous duplex arms to one another in the vicinity of a D-loop or HJ may promote CO formation by stabilizing early intermediates (such as SEIs) or imposing structural conformations required for hand-off to later pathway components. It is not known how many Msh4-Msh5 complexes are likely to be loaded at a designated CO site *in vivo*, but repeated Msh4-Msh5 loading and clamp sliding may be necessary or sufficient to enforce a local structural property along a homolog axis that (1) may explain CO resolution and (2) may partially account for CO interference. In other words, Msh4-Msh5 may contribute to the CO resolution bias for dHJs by defining their relative orientation such that incision



factors can only access each joint molecule in a manner that results in CO outcome.

2.9.3

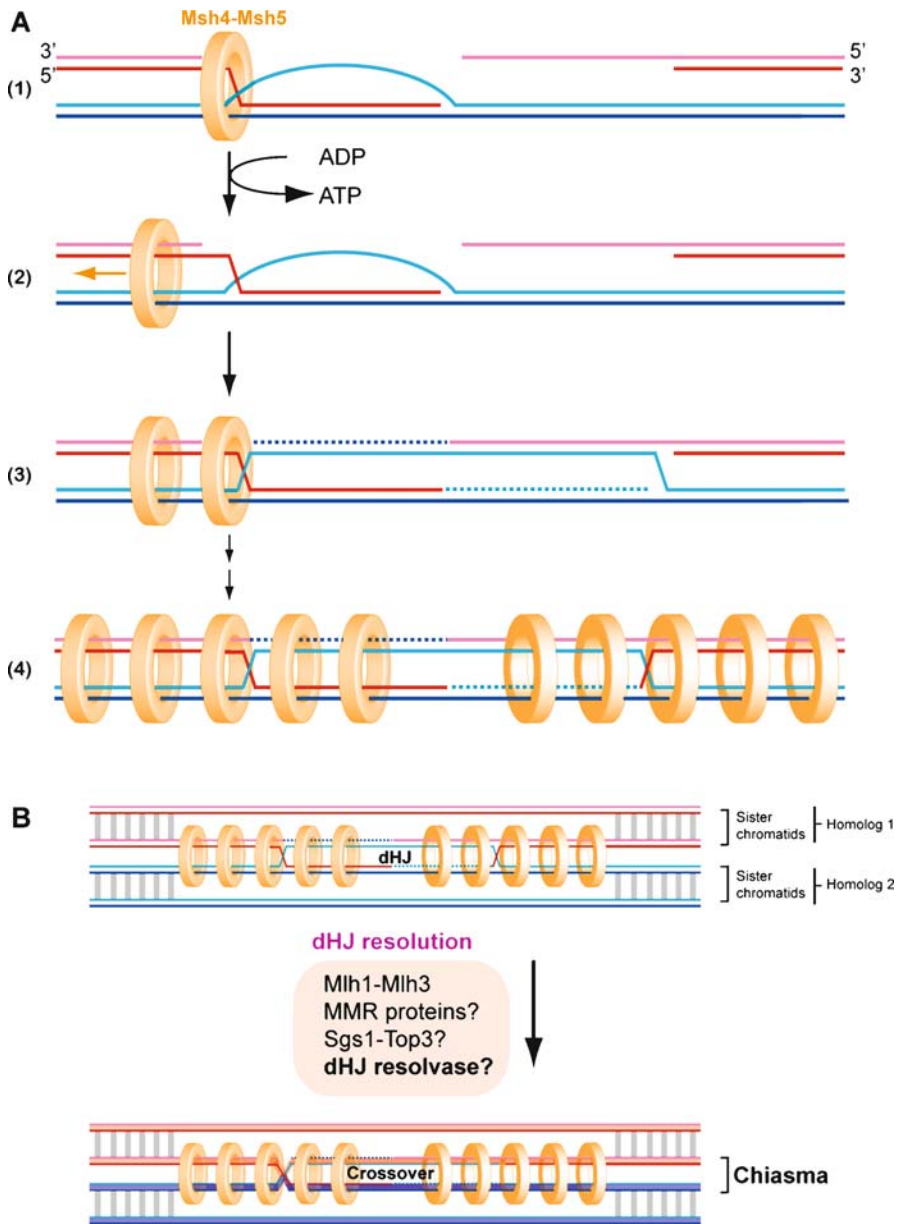
Mlh1-Mlh3: Working in Succession to Msh4-Msh5 During Crossover Formation

In addition to defining a putative DNA junction-specific homolog tether that promotes maturation along a CO pathway, Msh4-Msh5 is probably important

- ◀ **Fig. 8** Applications of mismatch repair to meiotic recombination. **A** Signals in the parental versus daughter DNA strands distinguish a newly replicated DNA strand from its template strand, and are used by mismatch repair proteins to target excision repair to the new strand and its misincorporated nucleotide. (1) Prokaryotic mismatch repair capitalizes on the hemimethylated state of newly replicated DNA; MutS (indicated as S) scans the DNA for discontinuities, to which it binds and in association with MutL (indicated as L), presents to the endonuclease MutH (indicated as H). (2) Eukaryotic mismatch repair, involving MutS and MutL homologs (MSH, MHL), identifies the newly replicated strand not based on an absent signal (absence of methylation), but more likely on the presence of a nick associated with Okazaki fragment processing during lagging strand DNA synthesis. As in prokaryotic MMR, the nick defines the starting point for an exonuclease that travels to the mismatched region and removes the misincorporated nucleotide(s). (3) DNA repair synthesis and ligation restores the helix. **B** The basic properties of mismatch repair (mismatch binding, structural recognition, DNA strand discrimination, and endonuclease instruction) may be employed for specific objectives of meiotic recombination. Several possibilities are shown here. (1) MMR factors bound to mismatches within heteroduplex DNA likely report on the degree of homeology or homology, which may distinguish ectopic from allelic targets. How MMR factors engage with a Rad51 or Dmc1 filament on hDNA is unknown and how they communicate to sanction hDNA extension or promote hDNA disruption is also unknown. (2) Repair of mismatches by MMR pathways is responsible for gene conversion. (3) The dHJ intermediate in budding yeast contains contiguous strands (Schwacha and Kleckner 1995), but a prior nick or another feature associated with DNA repair synthesis in CO pathway 1 may inform the placement of strand incisions by a dHJ resolvase, enforcing a CO outcome without a need for the two resolution events to be directly coordinated

for recruiting subsequent components of the CO pathway (Fig. 9). Genetic relationships between *msh4*, *msh5* and *mlh1*, *mlh3* plus analogy to the *E. coli* MutS-MutL system predict that Msh4-Msh5 heterodimers recruit Mlh1-Mlh3 heterodimers to the site of their structure-specific loading to DNA. *mlh1 msh4* and *msh4* mutants have a more severe phenotype than *mlh1*, suggesting that Mlh1 functions downstream of Msh4-Msh5 (Hunter 2007; Hunter and Borts 1997). Furthermore, Msh4 foci appear before Mlh1 foci in mice (Baker et al. 1996). *Mlh3*^{-/-} mice in turn are deficient in the formation of Mlh1 foci, indicating that Mlh3 recruits Mlh1 or that both proteins are needed for complex stability. What is the biochemical function of Mlh1-Mlh3?

Little is known about the biochemical properties of the eukaryotic complex, and most of what we anticipate for Mlh1-Mlh3 functions derives from analogy to the *E. coli* MutL homodimer, which coordinates factors such as helicases and nucleases downstream to mismatch recognition in MMR. Complexes containing Sgs1 and Mlh1-Mlh3 can be recovered from meiotic extracts, which may suggest a role for Mlh1-Mlh3 in recruiting the helicase/structure-migrating and decatenation activities of Sgs1-Top3 to CO-dedicated dHJs (Wang and Kung 2002). Unexpectedly, a latent endonuclease activity has been identified in the human MutL α heterodimer (Mlh1-Pms2; homologous to Mlh1-Pms1 in budding yeast) (Kadyrov et al. 2006). The nuclease active site was found in the Pms2 (*S. cerevisiae* Pms1) subunit, and the



sequence is conserved in Mlh3 but not Mlh1, Mlh2 or bacterial MutL. Incision of a nicked heteroduplex requires RFC, PCNA, MutS α (Msh2-Msh6) and ATP, and is directed primarily to the discontinuous strand for subsequent exonucleolytic removal of the region between the two nicks by Exo1 (Kadyrov et al. 2006). Presumably, the nicks that define the discontinuous strand in

- ◀ **Fig. 9** Msh4-Msh5 promotes crossovers by stabilizing joint molecules associated with single-end invasions. **A** Msh4-Msh4 loads at a joint (single-end invasion). An ADP → ATP switch induces Msh4-Msh5 to slide on duplex DNA, embracing both homologs within a ring-like complex. Iterative loading of Msh4-Msh5 complexes and their subsequent diffusion is thought to tether the homologs (Snowden et al. 2004). Loading at only one joint is shown, and it is unclear whether Msh4-Msh5 loads at the invasion intermediate or at both nicked Holliday junctions. Ligation of the nicked junctions results in covalently sealed dHJs, the intermediates presently believed to precede resolution to CO outcome in CO pathway 1 (Schwacha and Kleckner 1995). In association with Mlh1-Mlh3, MMR proteins, Sgs1-Top3, and/or an unidentified dHJ resolvase, dHJ resolution is directed or enforced to CO. **B** The Msh4-Msh5 dHJ and CO resolution to a chiasma is shown in the context of the bivalent; cohesion is depicted by gray lines and may also be rings encircling the chromatids rather than the homologs as by Msh4-Msh5. The loss of cohesion associated with Msh4-Msh5 loading on homologs may partially account for CO interference in CO pathway 1 (see Fig. 1), as loss of cohesion over long tracks associated with closely spaced Msh4-Msh5 COs may risk premature loss of sister chromatid cohesion

meiotic recombination derive from DNA strand exchange or second end capture directly (dsDNA–ssDNA junction in a D-loop or at an unligated Holliday junction), or may be associated with DNA repair synthesis. If endonuclease activity is confirmed for the Mlh1-Mlh3 complex, these intriguing observations suggest that not only have MMR functions of hDNA identification been co-opted for purposes of meiosis, but also that endonuclease activity associated with pathway members may figure in resolution of recombination intermediates. Whether a role in resolution is direct (in nick catalysis) or indirect (in guiding strand choice by an endonuclease) remains to be determined. At any rate, Mlh1 foci are the most consistent late markers for COs in mice, consistent with the mechanistic possibility that these MMR factors in some fashion direct resolution to CO.

2.10

Meiotic MMR

2.10.1

Mismatch Repair of hDNA and hDNA Rejection

Because the interacting DNA regions of heteroduplex are not identical nucleotide for nucleotide, hDNA is characterized by regions of unpaired bases. These mismatches may be dispersed at the single nucleotide level or occur over several bases as small bubbles. As substrates for meiotic MMR, these are the basis of gene conversion and fundamental to recombination models (Holliday 1964; Szostak et al. 1983) (see contribution by J.E. Haber, this BOOK; Fig. 8). The biochemistry underlying all MMR systems is well understood and based on a three-part logic: (1) a locally unpaired duplex region is bound by a protein complex that recognizes structural deformity associated with the mismatch, (2) a secondary protein joins the mismatch-bound complex, pre-

sumably stabilizing the first and communicating a further level of sanction that (3) recruits an endonuclease and instructs the position of its incision in one of the two duplex strands near the mismatch or utilizes a pre-existing nick for resection/resynthesis. Excellent reviews have covered the mechanistic details of MMR (Hoffmann and Borts 2004; Jiricny 2006; Modrich 2006; Schofield and Hsieh 2003). We will briefly allude to another role of MMR in quality control of recombination by rejection of hDNA, a critical function to avoid non-allelic interaction during meiotic recombination.

A mechanism to assess hDNA quality is presumably important to meiotic recombination, because only COs that occur at allelic positions form useful chiasmata that direct meiotic chromosome segregation. At non-allelic sites, COs pose a risk for inadvertent chromosomal translocations, inversions, or deletions. Moderate homology (homeology) is therefore not sufficient to authorize the maturation of a DNA strand invasion intermediate along a CO pathway. How is homology best detected during recombination, and how are off-target intermediates reversed or processed as NCOs?

Genetic experiments in bacteria and eukaryotes have clearly implicated MMR as a barrier to homeologous recombination, preventing ectopic interactions (Hunter et al. 1996) (reviewed in Schofield and Hsieh 2003). Biochemical experiments with the bacterial proteins have shown that MutS and MutL reduce the extent of hDNA formation by RecA and inhibit RuvAB-mediated branch migration (Fabisiewicz and Worth 2001; Worth et al. 1994). These data favor an hDNA rejection model, akin to D-loop dissolution during SDSA. However, how MMR communicates with the meiotic recombination machinery at the level of hDNA and which DNA helicases/translocases might be involved is unknown. The RecQ-like helicase Sgs1 is one likely candidate for a function at this stage (Myung et al. 2001).

2.10.2

Involvement of the Rad1-Rad10 Endonuclease in Meiotic MMR of Large Insertion/Deletions

Supplemental to the typical complement of MMR proteins, Rad1-Rad10 (XPF-ERCC1 in mammals) plays a specific role in the repair of long insertion/deletion loops during meiotic recombination (Kirkpatrick and Petes 1997). Rad1-Rad10 is required for the repair of loops that are 26 bases or more in size, and unpaired loops as large as 5.6 kb are compatible with assimilation into hDNA (Kearney et al. 2001). These observations suggest significant tolerance for insertions/deletions in heteroduplex so long as other requirements of homology are met in flanking regions. The conversion events associated with large deletions or insertions tend to restore sequence where there was a deletion. In other words, conversion is biased for the retention of sequence where there is disparity in a heteroduplex region. To achieve this bias, Rad1-Rad10 is proposed to cleave the bottom strand (as drawn in Fig. 10), opposite the

extruded ssDNA loop (Jensen et al. 2005). Incision at this position opens the loop so that its ssDNA becomes a gap flanked by duplex DNA. Rad1-Rad10 is a DNA structure-selective endonuclease complex best characterized for its role in the processing of UV photoproducts (nucleotide excision repair/NER) and in SSA (reviewed in Heyer et al. 2003). In NER and SSA, Rad1-Rad10 cuts 5' to a transition between dsDNA and ssDNA, where the ssDNA is directed 5' to 3'. Incision opposite the loop rather than adjacent to the loop therefore departs from the standard biochemical behavior observed for Rad1-Rad10 incision on DNA bubble substrates *in vitro*. Kearney et al. (2001) propose that RPA bound to the ssDNA loop directs Rad1-Rad10 incision to the bottom strand, rather than ssDNA in the loop. This unconventional incision site probably involves an additional interaction with the MMR complex Msh2-Msh3, which may promote Rad1-Rad10 incision appropriate to the long loop repair con-

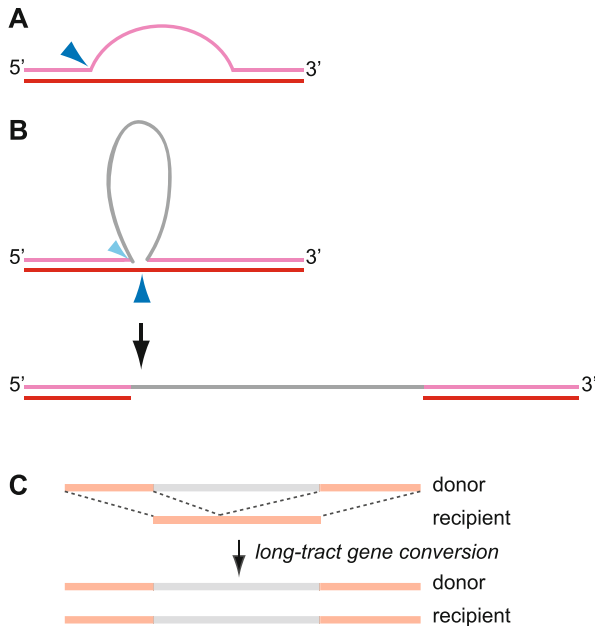


Fig. 10 XPF-ERCC1 promotes long-tract gene conversion by loop incision in a manner different from its incision during nucleotide excision repair. **A** XPF-ERCC1 is biochemically responsible for “upper-strand incision” 5' to a single-stranded DNA lesion during nucleotide excision repair (NER). RPA promotes cleavage of the bubble substrate. **B** In contrast to its behavior in NER, XPF-ERCC1 is proposed to incise the “bottom strand” of a looped substrate associated with a long-tract heterology during gene conversion (Jensen et al. 2005). This incision site is genetically implicated but remains to be biochemically demonstrated in association with the factors that might direct or promote its incision in this context (such as Msh2-Msh6). **C** The predicted incision site for XPF-ERCC1 in long-tract gene conversion promotes the preservation of long insertions in otherwise homologous sequences

text. Neither of these scenarios has been tested with a long loop substrate *in vitro*. This example illustrates how endonucleolytic incision might be dictated or sanctioned by the specific substrate context and other bound proteins.

In sum, MMR and long-loop repair processes account for the gene conversion products that are fundamental to meiotic recombination. MMR (and possibly long loop repair proteins) are also likely to perform key roles in communicating hDNA quality. How they achieve this for purposes of HR and its fidelity are questions that remain broadly open for biochemical exploration.

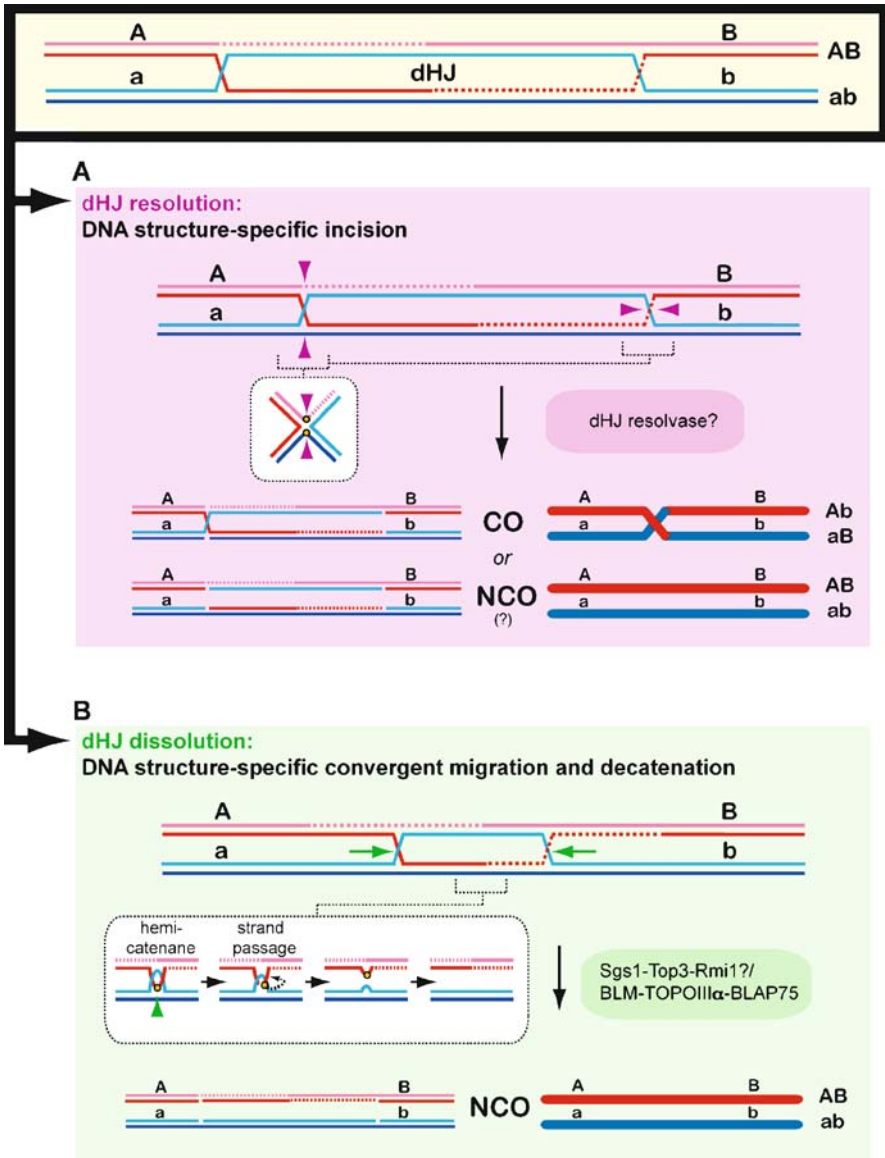
2.11

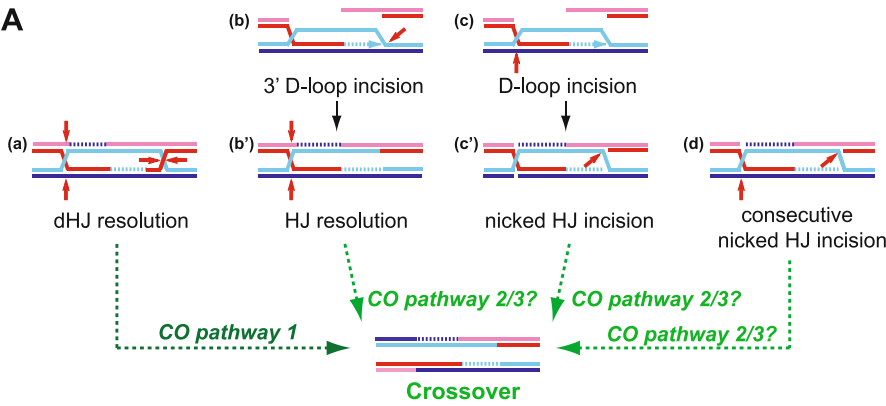
Double Holliday Junction Processing: Roads to Crossover and Non-Crossover

The dHJ is a key intermediate in the DSBR pathway of HR (Szostak et al. 1983) (Figs. 2, 11). Physical analysis of meiotic recombination intermediates in *S. cerevisiae* has demonstrated the existence of dHJs as paired and fully ligated interhomolog joint molecules consisting of contiguous strands, where the invading ends have been ligated (Schwacha and Kleckner 1994, 1995, 1997). Combined genetic and physical analysis defined a major meiotic CO pathway in *S. cerevisiae* (termed CO pathway 1, Fig. 12) that genetically depends on Msh4-Msh5, Mlh1-Mlh3, Exo1, and Mer3 (the Zip1-4 components are not discussed here; see for review Cromie and Smith 2007; Hunter 2007). This CO pathway posits the maturation of SEI intermediates into dHJs (Fig. 2), which appear to be dedicated to become COs that exert chiasma interference (Allers and Lichten 2001; Borner et al. 2004; Hunter and Kleckner 2001) (Figs. 1 part C, 12). This CO-specific model represents a significant deviation from the original DSBR model (Szostak et al. 1983), which envisioned an equal likelihood of dHJ resolution into CO and NCO products to account for roughly 50% association of COs with meiotic gene conversion. While endonucleolytic resolution has been consistently envisioned to

Fig. 11 Double-Holliday junction resolution and dissolution: CO and NCO outcomes. **A** dHJ resolution: dHJ incision by a DNA structure-specific endonuclease can yield CO or NCO outcome, depending on whether the same strands or different strands are cut at each junction. dHJs are recognized as intermediates of CO pathway 1 and are thought to be resolved primarily, if not exclusively, to CO outcome. *Inset*: HJ in open planar configuration, showing symmetric incision across its core. *Yellow bubbles* represent the two phospho-diester bonds hydrolyzed during HJ resolution. **B** dHJ dissolution: dHJ can be dissolved to NCO outcome by coordinated convergent migration of the Holliday junctions followed by their decatenation, a mechanism demonstrated for BLM-TOPOIII α -BLAP75. *Inset*: Single-strand DNA passage at a hemicatenane (collapsed dHJs) accounts for the separation to NCO. The *yellow bubble* represents the single phospho-diester bond hydrolyzed during DNA strand passage. Although the phospho-diester bond hydrolysis penultimate to strand passage probably occurs once at the single hemicatenane, topoisomerase nicking likely occurs repeatedly during dHJ convergent migration to relieve torsional strain associated with branch migration ►

resolve dHJs into CO or NCO products, recent biochemical experiments using the BLM DNA helicase in conjunction with TOPOIII α provided biochemical support for an alternative model of dHJ processing originally proposed to explain budding yeast mating-type switching (Nasmyth 1982; Wu and Hickson 2003). In a process termed dissolution, active migration of the two junctions towards each other and decatenation of the resulting hemicatenane yields





B

	Pathway 1	Pathway 2	Pathway 3
	Msh4-Msh5	Mus81-Mms4/Eme1	Mei-9 (XPF)-Mus312 *
<i>S. cerevisiae</i>	☒	☒	
<i>S. pombe</i>		☒	
<i>C. elegans</i>	☒		
<i>D. melanogaster</i>			☒
<i>A. thaliana</i>	☒	☒	
mammals	☒	?	
Interference	yes	no	yes

exclusively NCO products (Wu and Hickson 2003) (Fig. 11). While the genetic data in *S. cerevisiae* argue that a majority, if not all, of the meiotic dHJs are processed to COs, it is possible that dHJ dissolution to NCOs might be relevant for meiosis not only in the context of homolog but also sister interactions.

2.11.1
Sgs1-Top3-Rmi1 and dHJ Dissolution: Inferences from BLM-TOPOIII α -BLAP75

Extending earlier observations that BLM helicase can migrate model HJs (Karow et al. 2000), elegant biochemical studies of human and *Drosophila* BLM-TopoIII α on model dHJs demonstrate that the combined activities of the

- ◀ **Fig. 12** Pathways to generate crossovers. **A** Symmetric endonucleolytic resolution of dHJs (*a*) (Szostak et al. 1983) and single HJs *b'* (Holliday 1964) have long been considered to represent two mechanisms of generating crossovers. dHJ resolution represents CO pathway 1. CO pathway 2 is currently defined by Mus81-Mms4/Eme1, although the physical intermediates of this pathway are presently uncertain. A single HJ can be generated by endonucleolytic cleavage 3' to an extended D-loop (*b*), if the displaced strand of the D-loop reanneals with the second end *b'*. Two successive rounds of D-loop cleavage, or more specifically D-loop incision at the bottom strand (*c*) and nicked HJ incision *c'*, can generate a CO without the involvement of an intact HJ intermediate (Heyer et al. 2003; Osman et al. 2003). Finally, consecutive incision of nicked dHJs (*d*) can yield a CO product. **B** Taxonomic distribution of CO pathways. CO pathway 1 is defined by a collection of factors associated with Msh4-Msh5, including Mer3, Mlh1-Mlh3, and synaptonemal complex factors not discussed here. CO pathway 2 is defined by the XPF paralog Mus81-Mms4/Eme1, and CO pathway 3 by the XPF ortholog Mei9 (pathway 3). The size of the *checked box* indicates the relative use of the pathway in the given organism. (*) Mus81-Mms4/Eme1 and Mei9-Mus312 are the endonucleases responsible for the incisions believed to be associated with CO generation in CO pathways 2 and 3, but Msh4-Msh5 is not an endonuclease and the endonuclease responsible for dHJ resolution in CO pathway 1 remains to be identified. CO pathway 1 displays CO interference (see Fig. 1). Interference is not intrinsic to chiasmata, as COs in pathway 2 do not display interference (de los Santos et al. 2003; Munz 1994). In *Drosophila melanogaster*, most if not all COs depend on pathway 3 (Sekelsky et al. 1995), implying that COs in this pathway are associated with interference (Muller 1916; Sturtevant 1915)

BLM helicase and TOPOIII α topoisomerase can converge model dHJs and decatenate them to a NCO outcome (Plank et al. 2006; Wu and Hickson 2003). Work with human BLM-TOPOIII α on oligonucleotide-based dHJs demonstrated that dissolution activity was specific for an interaction between BLM and TOPOIII α , and no other helicase or topoisomerase can substitute in the relationship (Wu and Hickson 2003). dHJ dissolvase activity requires the catalytic activities of both proteins. ATP hydrolysis is required, suggesting that BLM helicase activity, and not DNA melting by binding alone, catalyzes convergent migration of the two HJs toward one another (Wu and Hickson 2003). This mechanism was authoritatively borne out by reactions with *Drosophila* BLM-TOPOIII α on large, circular dHJ substrates (Plank et al. 2006). Dissolution of these dHJs, separated by up to 165 bp of homologous sequence, demonstrated that BLM-TOPOIII α can migrate and decatenate the junctions without obligatory ssDNA. RPA stimulates the BLM-TOPOIII α reaction in a species-specific manner (the bacterial and T4 ssDNA binding proteins SSB and gp32 cannot substitute), suggesting that a functional complex entails BLM, TOPOIII α , and RPA (and therefore that some measure of ssDNA is generated by the complex and involved in the reaction mechanism). Further support for the role of convergent migration in dHJ dissolution was supplied by limiting junction migration in the model substrates. The bacteriophage HJ resolvase T7 endo I processes dHJs in the presence of interstrand crosslinks, but BLM-TOPOIII α -RPA dissolution is inhibited (Plank et al. 2006). Additional substrates that were

engineered with discrete mismatches underwent exchange of complementary strands to generate novel restriction sites. These observations suggest that duplex DNA is unpaired ahead of the migrating junctions and re-annealed behind them, consistent with convergent migration of the junctions toward one another. In reactions with *Drosophila* BLM-TOPOIII α , up to 30 linkages between the dHJs were dissolved. This indicates a mechanism in which the complex resides at each HJ and performs iterative strand passage events at each step position of convergent migration (Plank et al. 2006).

At present, it is not clear how a directionality of HJ migration compatible with dissolution is enforced, because in principle the junctions can be migrated toward or away from one another. One possibility relates to a factor that may target BLM-TOPOIII α to dHJs. Rmi1 (BLAP75/RMI1 in mammals) is an OB-fold protein identified as a coimmunoprecipitating member of Sgs1-Top3 and BLM-TOPOIII α complexes (Chang et al. 2005; Mullen et al. 2005). Human BLAP75/RMI1 and budding yeast Rmi1 bind weakly to ssDNA on their own, but stimulate Top3 ssDNA binding by approximately fivefold and enhance Top3-catalyzed relaxation of supercoiled DNA (Chen and Brill 2007; Wu et al. 2006a). dHJ dissolution by human BLM-TOPOIII α under conditions of physiological ionic strength depends on BLAP75/RMI1 (Raynard et al. 2006), highlighting a specific relationship not observed with TOPOIII α and WRN or *E. coli* RecQ (Wu and Hickson 2003). Whether BLAP75/RMI1 promotes a conformational change in TOPOIII α -BLM that promotes DNA binding, or whether it helps melt DNA structural regions that enhance TOPOIII α binding, is unclear. How BLAP75/RMI1 promotes the helicase-topoisomerase cooperation for convergent migration of dHJs is also unclear, although it could possibly determine the orientation with which the complex loads on the DNA.

dHJ dissolution has not been demonstrated for *S. cerevisiae* Sgs1-Top3 due to the challenges in purifying the proteins. Whether Rmi1 is needed for or enhances Sgs1-Top3 activities is also not known. Nevertheless, the observations that mutations in *rmi1* mimic those of *sgs1* and *top3* strains for slow growth, hyper-recombination, reduced sporulation and genotoxin sensitivity indicate that a potential targeting factor may be absolutely required for the *in vivo* execution of a biological activity under normal protein expression levels (Mullen et al. 2005).

Does dHJ dissolution explain the genetic requirements for Sgs1-Top3 during meiosis? Genetic observations for Sgs1 in mitotic cells are consistent with a role in CO suppression, and/or promotion of NCO dissolution of recombination intermediates. Whether the NCO contribution *in vivo* is by dHJ dissolution, however, is difficult to deduce and not likely to be the only mechanism by which Sgs1-Top3 promotes NCO. Ira et al. (2003) observed in an ectopic DSB-induced recombination assay that COs were rare in mitotic cells (5%) and that disruption of *sgs1* increased CO two- to threefold. Absence of Sgs1 did not affect the DSB repair efficiency or kinetics, suggesting that Sgs1 does not curtail DSB repair events but influences their outcome to re-

sult in NCOs (Ira et al. 2003). These genetic data fit well with the proposal that Sgs1-Top3 functions in the dissolution of dHJs in vegetative *S. cerevisiae* cells (Ira et al. 2003), although the existence of dHJs in mitotically growing cells has not been physically demonstrated yet. Sgs1-deficient cells also display elevated CO frequencies in meiosis, although the effect is rather subtle (<twofold) (Jessop et al. 2006; Oh et al. 2007; Rockmill et al. 2003). An early role of Sgs1 in meiotic recombination may be in the turnover of pairing intermediates generated during a mechanism that queries homology (Myung et al. 2001). A role for Sgs1 in hDNA rejection during SDSA in vegetative cells has also been described (Sugawara et al. 2004). Consistent with this possibility, human BLM promotes dissolution of D-loops, as mentioned earlier (Bachrati et al. 2006; Bugreev et al. 2007b; Orren et al. 2002; van Brabant et al. 2000) (see above Sect. 2.6). Like Mer3, BLM helicase has a 3' $\leftrightarrow$ 5' polarity for translocation, but presumably a different loading position achieves heteroduplex disruption rather than extension (see Sect. 2.9; Fig. 7). The role of Sgs1 in meiotic CO suppression has been further clarified by genetic analysis of double mutants and physical analysis of recombination intermediates. Mutants in the CO pathway 1 of *S. cerevisiae* (Fig. 2; SEI-dHJ), in particular *mlh3* and *msh5*, show a significant decrease in CO, which is completely suppressed by Sgs1 deficiency (Jessop et al. 2006; Oh et al. 2007). This could suggest that the dHJ clamp Msh4-Msh5 protects the dHJ from dissolution by Sgs1, in support of the notion that dHJs are CO-dedicated intermediates (Allers and Lichten 2001; Borner et al. 2004; Hunter and Kleckner 2001). Careful genetic analysis identified that Sgs1 specifically suppresses closely spaced double COs, and that the increase in COs in the *sgs1* mutant can be primarily explained by a transition from single COs to closely spaced double COs (Oh et al. 2007). Physical analysis of the recombination intermediates provided a mechanistic basis for this genetic outcome, by showing that the two ends of the DSB become discoordinated in *sgs1* cells, leading to the accumulation of joint molecules with three or four partners at the expense of joints with the canonical two partners (Oh et al. 2007). This function of Sgs1 is unlikely to be explained by dissolution of dHJs generated by second end capture, but rather pertains to the independent joints formed by the second end.

Do all meiotic functions of Sgs1 take place in association with Top3, or are only specific functions associated with the strand passage activity of the topoisomerase? This question is difficult to answer at the moment. Top3 function is essential in *S. cerevisiae* meiosis, and the phenotypes of the *top3* mutant are more extreme than those of an *sgs1* mutant. Few *top3* cells are able to complete meiosis I, and spore viability is zero. However, physical analysis reveals that DSB levels and the kinetics of DSB processing at the *CYS3* and *ARG4* meiotic recombination hotspot loci resemble wild-type in the *top3* mutant, suggesting that the defect occurs late and may entail HJ resolution or dissolution (Gangloff et al. 1999). Rather than mirroring the *top3* phenotype for meiosis, inactivation of *SGS1* in fact relieves the severity of the *top3* de-

fect (spore viability of *sgs1* is the same as *sgs1 top3*, at 67%). Also, Top3 may function outside the context of the Sgs1-Top3-Rmi1 complex.

In sum, dHJs are concrete intermediates of the CO pathway 1 in *S. cerevisiae*, but details of their biochemical processing in meiosis remain a crucial deficiency in our understanding of eukaryotic meiotic recombination (see also below). The original proposal of the DSBR model, that CO and NCO are alternative outcomes of dHJ resolution, can no longer be maintained. dHJs develop from stable SEI intermediates, which are determined from early strand exchange to mature along a CO-designated pathway. These observations make less certain the role of dHJ dissolution by Sgs1-Top3-Rmi1 in meiosis, although this mechanism may present an alternative to SDSA in the generation of NCO from intermediates aborted early in meiosis or in sister interactions.

2.11.2

Double Holliday Junction Resolution: The Holy Grail and Resolvase A

Endonucleolytic resolution of HJs is a central tenet of recombination models to explain CO formation (Holliday 1964; Szostak et al. 1983) (Figs. 2, 11). The physical demonstration of their existence and the identification of enzymes that are highly selective for HJs in bacteria (RuvC), Archaea (Hjc, Hje), and eukaryotic mitochondria (Cce1) provided experimental support for this model (reviewed in Heyer et al. 2003; West 1997). In particular, the elegant work on bacterial RuvC has set a paradigm for a eukaryotic nuclear HJ resolvase and its reaction mechanism of delivering a symmetric pair of cuts across the junction that allows direct religation of the products (West 1997). None of the known HJ resolvases has an obvious sequence homolog in eukaryotes. However, such a relationship may be difficult to discern. As an example, the similarity between archaeal Hjc and the eukaryotic XPF family, and their evolutionary relationship to eubacterial type II restriction endonucleases, was not recognized until structural comparisons revealed a common architecture and configuration of a small set of active site residues (Bond et al. 2001; Nishino et al. 2003). This suggests that resolvases with similar architecture to bacterial or archaeal HJ resolvases cannot be categorically discounted in eukaryotic genomes from sequence analysis alone. The identity of the eukaryotic nuclear resolvase is still unknown, although significant progress in this direction has been made for the mammalian Resolvase A activity.

A mammalian activity that symmetrically cuts model HJs has been partially purified from Chinese hamster ovary cells, human HeLa cells, and several DNA repair-deficient cell lines (Constantinou et al. 2001, 2002; Hyde et al. 1994; Liu et al. 2004). Fractionation of cell extracts over several columns consistently yields activity that generates duplex products that can be religated. In a compelling analogy to the RuvABC paradigm, the incision activity copurifies extensively with an ATP-dependent junction-migration activity

(Constantinou et al. 2001). Nevertheless, the protein factor(s) responsible for branch migration and DNA incision continue to elude identification. The incision activity was called Resolvase A and can be separated from Mus81-Mms4/Eme1, a DNA structure-selective endonuclease also implicated in the processing of recombination-associated joint molecules (see Sect. 2.12. Resolvase A shows a greater preference for HJ structures relative to the three-way, replication fork-like structures preferred by Mus81 (Constantinou et al. 2002). Most recently, it was reported that the Rad51 paralogs Rad51C and Xrcc3 were required components of the HJ incision associated with a partially purified mammalian fraction (Liu et al. 2004). Immunodepletion of Rad51C from extracts reduced model HJ incision in vitro, but addition of recombinant Rad51C restored junction cleavage. Recombinant Rad51C-Xrcc3 was devoid of resolvase activity, suggesting that the endonuclease activity is associated with a different protein. Furthermore, Rad51C localizes to the obligatory CO site during XY pairing in mouse cells, in a temporal sequence that follows Mlh1 arrival (Kuznetsov et al. 2007; Liu et al. 2007) (see Sect. 2.9.3). These data may be consistent with a late role for Rad51C-Xrcc3 associated with meiotic CO sites, but a mechanistic explanation to link Rad51C-Xrcc3 to the resolvase responsible for resolution in vitro and in vivo is still needed.

2.12

Other Junctions and Alternative Mechanisms for Crossover Formation: Possible Roles of Mus81-Mms4 and XPF

dHJs are the key intermediate for the meiotic CO pathway 1 of *S. cerevisiae* that depends on Mer3, Msh4-Msh5, Mlh1-Mlh3, and other proteins (Figs. 11 and 12). However, disabling this pathway reduces CO frequencies by only about twofold in budding yeast. A second meiotic CO pathway in budding yeast is defined by the Mus81-Mms4 endonuclease. There is significant evidence, discussed below, that this pathway does not involve dHJs in the formation of COs. The fission yeast *S. pombe* lacks CO pathway 1 and the proteins associated with its mechanism; most, if not all, meiotic COs depend on Mus81. As a mirror image, *Caenorhabditis elegans* exclusively relies on CO pathway 1 for meiotic CO formation (Zalevsky et al. 1999) as all COs appear to depend on Msh4-Msh5. Nonetheless, this organism has a Mus81 homolog but genetic analysis of the *C. elegans mus81* mutant in meiosis remains to be reported (Interthal and Heyer 2000) (for review Hollingsworth and Brill 2004). In *Drosophila*, meiotic COs are largely controlled (>90%) by yet another pathway, involving the XPF (*Drosophila mei-9*) endonuclease function. *Drosophila mus81* mutants show no meiotic CO defect (Trowbridge et al. 2007), and crossover pathway 1 may be absent, as indicated by the lack of Msh4/Msh5 homologs in *Drosophila* (Sekelsky et al. 2000a) (Fig. 12). Clearly, alternatives to dHJ resolution-mediated CO formation exist (Fig. 12), but what are the enzymes involved?

2.12.1

The Structure-Selective DNA Endonuclease Mus81-Mms4/Eme1

Mus81-Mms4/Eme1 is a well-conserved heterodimeric XPF family DNA endonuclease consisting of a catalytic subunit (Mus81) and a non-catalytic subunit. (Mms4 in budding yeast/Eme1 in fission yeast and mammals; therefore the complex is frequently referred to as Mus81-Mms4/Eme1. In this text, "Mus81" is also used to refer to the complex from any organism.) Mus81 functions as a context-specific factor in recombination in vegetative cells and defines the meiotic CO pathway 2 in budding yeast and the major, if not only, CO pathway in fission yeast (reviewed in Cromie and Smith 2007; Heyer et al. 2003; Hollingsworth and Brill 2004). Mus81 was identified by a series of genetic screens that implicated the protein in recombination associated with replication fork support. Mus81 was initially isolated in *S. cerevisiae* by a two-hybrid screen for proteins interacting with the Rad54 motor protein and was shown to be a member of the *RAD52* epistasis group, although the single mutant lacks sensitivity to DSBs that typifies this group (Interthal and Heyer 2000). Independently, Mus81 was identified in a synthetic lethal screen with *sgs1*, demonstrating the need for the Mus81-Mms4 complex in cells lacking Sgs1, Top3, or Rmi1 (Mullen et al. 2001). In fission yeast, Mus81 was also found as a two-hybrid interactor with the S-phase checkpoint kinase Cds1 (Rad53 in budding yeast), and shown to be particularly required for replication fork support (Boddy et al. 2000). While Mus81 plays an important role in recombination pathways that support DNA replication, the discussion here will focus on its role during meiotic recombination.

What is the substrate by which the endonuclease activity of Mus81 promotes meiotic CO? One clear possibility is that Mus81-Mms4/Eme1 cleaves HJs. Biochemical studies suggested that the endogenous fission yeast and human Mus81 complexes resolve model HJs to linear duplex products, with incision targeted to the homologous core of the structure (Boddy et al. 2001; Chen et al. 2001; Gaillard et al. 2003). However, HJ incision has been difficult to observe for recombinant enzyme from any organism and, where observed, has been primarily restricted to incision by partially purified preparations from eukaryotic expression sources (Blais et al. 2004; Boddy et al. 2001; Chen et al. 2001). In fact, biochemical studies with budding yeast, fission yeast, and human enzyme complexes produced and isolated from expression in *E. coli* mostly show negligible incision on model HJs in vitro (Doe et al. 2002; Gaskell et al. 2007; Kaliraman et al. 2001; Whitby et al. 2003) (reviewed in Haber and Heyer 2001; Hollingsworth and Brill 2004). At first this was attributed to a possible need for post-translational modifications to the complex that occur only in eukaryotic expression sources but not bacterial sources, or to a need for an unidentified cofactor present in partially purified eukaryotic preparations and not in homogenous recombinant preparations. However, purified preparations of budding yeast Mus81-Mms4 from the cognate host and with endogenous

modifications are also not competent in HJ incision in vitro (Ehmsen and Heyer 2008). Given that nicked substrates are processed much more readily than intact junctions, Gaillard et al. (2003) proposed that endogenous Mus81-Mms4/Eme1 resolves HJs by a nick-counterneck mechanism. The first incision is proposed to be rate-limiting, but when successful, directs subsequent incision across the branch point. The incongruity in HJ activity by endogenous and recombinant preparations remains to be satisfactorily explained.

Aside from the difficulty in demonstrating robust HJ incision activity, model HJ cleavage by Mus81 under certain in vitro conditions takes place in a manner unlike HJ resolvases characterized to date. RuvC shows coordinated incisions on opposing strands of like polarity, in a manner that generates defined nicks in the product duplexes (West 1997). The product nicks are substrates for ligase in the final step of repair, indicating that the two phosphodiester bonds hydrolyzed during junction resolution occurred symmetrically across the junction core. Analysis of Mus81 HJ incision products suggests that the cuts are offset, because only a small fraction of the incisions can be directly ligated (Boddy et al. 2001; Constantinou et al. 2002; Gaskell et al. 2007). At least three possibilities can account for these observations, but the explanation is unclear with the present data sets: (1) the criteria to call an enzyme a HJ resolvase may need to be reframed, (2) the in vitro preparations are missing components important to the placement of the endonuclease and enforcement of symmetric incisions, or (3) the HJ incision observed represents the uncoordinated products of two independent incision events. Since the cell is equipped to resolve gaps and flaps, an asymmetric mechanism of HJ resolution cannot be dismissed categorically.

Quantitative substrate analysis with the Mus81-Mms4/Eme1 heterodimer demonstrated that the nuclease is not DNA structure-specific in vitro, but rather is structurally *selective* for substrates that have a branch point coincident with a strand discontinuity (nick) (Ehmsen and Heyer 2008). The biochemical preferences of Mus81 preparations in vitro are consistent with a number of substrates that may be valid physiological alternatives to intact HJs. 3'-flapped structures, D-loop structures, nicked three-way junctions, various replication fork-like substrates, and nicked four-way junctions are processed efficiently by all heterodimer preparations (Doe et al. 2002; Ehmsen and Heyer 2008; Fricke et al. 2005; Gaillard et al. 2003; Gaskell et al. 2007; Kaliraman et al. 2001; Osman et al. 2003; Whitby et al. 2003). Mus81-Mms4 incision of joint molecules appears to take place five nucleotides 5' to the end of a duplex flush with the branch point, suggesting that the 5' position of a nick in the DNA is a defining feature that directs Mus81 incision (Bastin-Shanower et al. 2003).

Evidently, it is difficult to extrapolate the biochemical behavior of Mus81 to a most likely in vivo joint molecule substrate. Are genetic observations consistent with HJ resolution? *S. cerevisiae* *mus81* and *mms4* mutants show a modest (twofold) reduction in meiotic CO, and 40% spore viability (de los Santos et al. 2001, 2003; Interthal and Heyer 2000). Physical analysis of recom-

bination intermediates in *mms4* cells identified a reduction in SEI and dHJ intermediates, which define the meiotic CO pathway 1 (de los Santos et al. 2003). Hence it is unlikely that Mus81 processes these intermediates. Physical analysis failed to identify other junction intermediates that accumulate in *mms4* mutants and might otherwise have revealed its *in vivo* substrate. These data suggest a biochemical role for Mus81 that is independent of dHJs. CO formation can be achieved in ways that do not involve dHJs, for example by consecutive incision of D-loops results in CO formation (Heyer et al. 2003; Osman et al. 2003) (Fig. 12). A D-loop/nicked HJ model combines the *in vitro* substrate preference of Mus81-Mms4 for D-loop and nicked four-way junctions with a genetic outcome that always leads to CO (Fig. 12).

In severe contrast to *S. cerevisiae*, the meiotic phenotype of fission yeast *mus81* and *eme1* mutants shows a massive failure to segregate chromosomes due to unresolved recombination intermediates (Boddy et al. 2000; Osman et al. 2003). Spore viability is reduced to less than 1% (relative to 80% in wild-type cells), and most DNA is relegated to one large spore product with three small spores nearly devoid of DNA. Total CO is reduced 20- to 100-fold (Osman et al. 2003; Smith et al. 2003). This DNA segregation defect contrasts the phenotype of *rhp51* mutants, which accumulate DSBs but nevertheless segregate DNA rather equitably to the four spore products. When Spo11-catalyzed initiation of meiotic recombination is blocked by additional mutations in *rec6* or *rec12*, viability of *mus81* mutants is improved to 20%, a value that is close to that expected from random segregation in fission yeast meiosis (Boddy et al. 2000). Genetic analysis in fission yeast shows that *mus81* mutants generate heteroduplex DNA and achieve normal levels of gene conversion, but with severely reduced CO levels (19–90% reduction over three genetic intervals tested) (Osman et al. 2003; Smith et al. 2003). This level of spore viability is less than expected for random segregation of three homologs at meiosis I. Together these observations make a compelling case for a late role for Mus81-Mms4/Eme1 and that recombination intermediates accumulate in *mus81* mutants that impede chromosome segregation. Physical analysis of recombination intermediates during fission yeast meiosis identified single HJs as a prominent class, and the authors suggested that single HJs rather than dHJs are the key intermediates for CO formation in fission yeast (Cromie et al. 2006). The accumulation of single HJs in *mus81* mutants is consistent with a single HJ being a Mus81 substrate, although it is also possible that the Mus81 substrate is different but is processed to an HJ in the mutant. While the biochemical data strongly favor a nicked HJ as a potential Mus81 substrate, the genetic data imply that the observed junctions are likely to be unnicked. Fission yeast has relatively rare DSB sites but an evenly spaced genetic map, which appears to mandate that CO-dedicated junctions migrate over significant distances from the DSB initiation site (Young et al. 2002). Such branch migration of HJs would remove possible nicks left from their initiation as DNA strand exchange products.

In sum, it is clear that Mus81-Mms4/Eme1 catalyzes CO formation in a subpathway of meiotic recombination in budding yeast, which is apparently the primary or exclusive pathway of meiotic recombination in fission yeast. What remains unclear is the identity of the substrate underlying the CO outcome. Although this may ultimately find more unequivocal support in the form of a traditional HJ, it is very possible that an alternative junction, characterized by a nick inherent to the branch point, may be the substrate in question for Mus81-Mms4/Eme1.

2.12.2

XPF Controls Meiotic Crossovers in *Drosophila*

Drosophila shows reliance on neither the Mus81 nor Msh4-Msh5 pathways for meiotic CO formation, but instead relies on its XPF ortholog, Mei-9 (Sekelsky et al. 1995; Yildiz et al. 2004). Mei-9 is required for 90–95% of CO in meiosis, very different from budding and fission yeast where the XPF orthologs are not involved in meiotic CO formation (Fleck et al. 1999; Paques and Haber 1999; Sekelsky et al. 1995; Yildiz et al. 2004). *Mei-9* disruption depletes COs, but not gene conversion, suggesting that it acts at a late recombination step that may be HJ resolution (Radford et al. 2007). In nucleotide excision repair and SSA, Mei-9 interacts with ERCC1 (Sekelsky et al. 2000b). In meiosis, however, Mei-9 also associates with a novel partner, Mus312 (Yildiz et al. 2002). Surprisingly, *ercc1* mutants are not as CO-defective as either *mei-9* or *mus312*, suggesting that Mei-9 may partner with alternative subunits in different contexts (Radford et al. 2005). Although it is still relatively unclear how ERCC1 contributes to the enzymatic activity or structural selectivity of the XPF-ERCC1 complex, ERCC1 binds ssDNA and may define substrate recognition and protein interaction properties of the complex (Tsodikov et al. 2005). Mus312 may similarly target Mei-9 to a branched DNA structure or alter HJ presentation to Mei-9 (or Mei9-ERCC1) to effect junction resolution. This scenario resembles BLAP75/RMI1 targeting of BLM-TOPOIII α to a DNA joint molecule and may provide another example of a manner by which the *in vivo* selectivity of a catalytic entity is narrowed to a specific application in sanctioned contexts, by association with a context-specific partner subunit. Whether the *Drosophila* situation reflects a third mechanism for CO formation (distinct from either the Msh4-Msh5 or Mus81-Mms4/Eme1 mechanisms) or merely the application of a different XPF paralog to a similar mechanism as Mus81-Mms4/Eme1 (more basically an “XPF paralog” mechanism) remains to be better defined (Fig. 12). However, unlike the Mus81-dependent CO pathway 2, which lacks interference in fission yeast (Munz 1994) and budding yeast (de los Santos et al. 2003), the Mei-9 pathway in *Drosophila* generates COs with interference (Muller 1916; Sturtevant 1915), and hence is termed CO pathway 3 here.

What is the substrate for Mei-9 in CO generation – is it possibly the substrate most frequently implicated in a CO defect, the HJ? Genetic experiments

led to the proposal that Mei-9 acts on dHJs in *Drosophila* meiosis, but the physical nature of these junctions (nicked or ligated dHJs) remains undetermined (Radford et al. 2007). Although biochemical analysis of Mei-9 remains to be reported, extrapolations from XPF orthologs may suggest HJ incision is at least available as a biochemical option. In a biochemical role distinct from its incision at dsDNA–ssDNA transitions, *S. cerevisiae* Rad1 (XPF homolog) can bind and incise model HJs in vitro (Habraken et al. 1994). Junction binding and incision occurred even in the absence of Rad10 (ERCC1 homolog) and was not stimulated by Rad10. Although Rad1 bound a model HJ whether it had a homologous core or not, it incised only junctions with a homologous core and did so over a number of asymmetric positions near the core. It was suggested, however, that this in vitro behavior of Rad1 and its dependence on a homologous core was an artifact of local “breathing” at the branch point that presented a dsDNA–ssDNA transition for Rad1 incision (West 1995). Alternatively, XPF-ERCC1 (Mei-9-Mus312 +/- ERCC1) may process D-loops in a fashion similar to its proposed role in gene targeting (Niedernhofer et al. 2001), which will result in obligatory COs (see Fig. 12). D-loop cleavage may be an activity common to XPF paralogs in certain contexts, and may represent a means to produce COs in a mechanism that does not involve HJs.

3

Conclusions and Outlook

Much progress has been made in identifying and characterizing core recombination factors and some of the meiosis-specific components of the HR machinery. However, the mechanistic basis for the features that distinguish meiotic recombination from recombination in vegetative (mitotically cycling) cells remains unknown. Genetic and cytological data provide a framework to unravel the mechanisms involved in the targeting of meiotic recombination to homologs, as well as the control of CO levels and distribution (Cromie and Smith 2007; Gerton and Hawley 2005; Hunter 2007; Roeder 1997; Zickler and Kleckner 1999). These analyses suggest a central role of Rad51 and Dmc1 (and its meiosis-specific cofactors) to bias DNA strand invasion towards the homolog, but the biochemical basis for this bias remains to be determined. As an additional mechanism that was not discussed here but is pertinent, a meiosis-specific barrier to intersister recombination is envisioned that involves the meiosis-specific proteins Red1 and Hop1 that control the meiosis-specific checkpoint kinase Mek1 (see also Niu et al. 2005; Wan et al. 2004). The roles of Red1 and Hop1 as well as the potential targets of Mek1 that could be involved in establishing the intersister barrier remain to be determined (for a detailed discussion see Hunter 2007). It appears that a combination of meiosis-specific aspects of the recombination machinery

and as yet poorly understood structural aspects of meiotic chromosomes will be involved.

Beyond the biochemical mechanisms that guarantee DNA strand exchange between homologs in the meiotic bivalent, many questions remain for how the resulting DNA joint molecules are processed to ensure a CO exchange. Most of these questions concern how apparently undifferentiated precursors are processed for dissimilar fates. This question of precursor fate appears at the earliest stages of recombination at a given locus – how is a single chromatid chosen for the Spo11-catalyzed break and what ensures that only one chromatid of four receives this fate? Almost immediately, a question of fate choice resurfaces for the development of DSBs to CO or NCO pathways. Genetic evidence supports the operation of at least two CO pathways in budding yeast, one defined by the consecutive actions of Mer3, Msh4-Msh5, and Mlh1-Mlh3, and another defined by Mus81-Mms4/Eme1. How is a DSB selected for maturation along one of these pathways? At what point do the different pathways diverge, and what are the deciding factors and contexts that determine their fates? How is CO distribution and interference achieved in the Msh4-Msh5 pathway? What is the *in vivo* substrate and mechanism for CO in the Mus81-Mms4/Eme1 and Mei9 pathways? What is the identity of the resolvase expected to process the dHJ intermediates of the Msh4-Msh5 pathway? An explanation for the taxonomic distribution of these CO pathways is needed, as is an understanding of whether the different pathways apply to special circumstances of chromosome structure. Finally, the specialized contributions of MMR proteins in meiotic recombination are poorly understood. Some possibilities for biochemical query include hDNA quality detection, the designation of strand exchange intermediates to NCO or CO resolution pathways, and the direction of strand incisions to catalyze CO at dHJs. Models for meiotic recombination have been updated with the understanding that several pathways process Spo11-induced breaks, and that NCO and CO designations occur far earlier than once anticipated. Now challenges remain to further characterize the newly recognized pathway variations, their underlying DNA structural intermediates, and their biochemical processing.

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Cover

The cover illustration depicts two key events of DNA repair: 1. The ribbon model shows the structure of the termini of two Rad50 coiled-coil domains, joined via two zinc hooks at a central zinc ion (sphere). The metal dependent joining of two Rad50 coiled-coils is a central step in the capture and repair of DNA double-strand breaks by the Rad50/Mre11/Nbs1 (MRN) damage sensor complex. 2. Immunolocalization of histone variant γ -H2Av in γ -irradiated nuclei of *Drosophila* germline cells. Fluorescent foci indicate one of the earliest known responses to DNA double-strand break formation and sites of DNA repair.

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Preface

This book concludes our tandem edition on *Recombination and Meiosis*. Subtitled *Models, Means and Evolution*, it follows its first-born twin with emphasis on *Crossing-Over and Disjunction*. In the commissioning of chapter topics we have tried to cover numerous aspects of the meiotic system from many different angles.

Both these books are embedded as volumes 2 and 3 in a topical Series devoted to *Genome Dynamics and Stability*, where DNA transmission and maintenance functions are discussed from experimental and theoretical perspectives. The earlier vol. 1 dealt with *Facets and Perspectives of Genome Integrity*, focusing on DNA damage repair mechanisms, and an upcoming vol. 4 is on transposable elements. These books on meiotic processes, together with other volumes in this Series on genome management in mitotic cells, provide a grass-roots level starting platform—initiating a prospective trajectory superimposable upon the exploding field of molecular cell physiology, or *systems biology* (see below).

The preceding volume preferentially dealt with meiotic processes in multicellular organisms, such as plants and animals including man. Also, basic accomplishments from work on yeasts was presented in a comparative perspective—concerning the decisive roles of Spo11-induced breaks for crossing-over, of sister chromatid cohesion in chromosome disjunction, and cell cycle modulation in the global control of the meiotic program. The present book puts additional focus on yeasts as unicellular model organisms, where progress in revealing the mechanisms of meiotic recombination has taken place most rapidly and systematically. Also, a central aspect of genetic recombination in *E. coli* is included for its outstanding merits as a universal model. Furthermore, three facets of evolutionary relevance are also discussed.

As for the models and means of meiotic recombination, two prominent and comprehensive chapters call for particular attention. Inasmuch as theoretical interpretations of empirical data about the exchange of genetical markers in successive generations has long preceded their biochemical elucidation, James E. Haber gives expert guidance on a veritable tour de force, presenting the *Evolution of Recombination Models* from purely genetic crosses into the molecular era. He follows the historical record from simplistic breaking/joining schemes to break-induced replication, from suspected single-strand breaks to partner

choice by single-strand annealing, and from the generation of double-strand breaks (DSBs) to their repair by the establishment and resolution of single or double Holliday junctions, and finally to DSB repair in the absence of crossing over accomplished through synthesis-dependent strand annealing that does not involve Holliday junctions. This scenic ride is aptly complemented from the enzymatic perspective, as displayed by Kirk T. Ehmsen and Wolf-Dietrich Heyer on the *Biochemistry of Meiotic Recombination: Formation, Processing, and Resolution of Recombination Intermediates*. These authors highlight the biochemistry of meiotic recombination, as more and more meiosis-specific enzymes have been added to the basic toolbox, which likewise is at work in mitotic cells (cf. GDS vol. 1, this SERIES). Overlapping with functions in replication and DSB repair these enzymes¹ comprise topoisomerase, nuclease, recombinase, polymerase, and helicase activities, as well as single-strand stabilizing protein, a protective end-tethering complex and a range of modulating co-factors.

The single most remarkable feature about the initiation of meiotic recombination is the deliberate and catalyzed introduction of numerous DSBs in the chromosomal DNA. Notably, the enzyme responsible for this pivotal and conserved activity is derived from a former topoisomerase (Spo11; Keeney, this SERIES), which as such had a cell-intrinsic function essential for the untangling of replication intermediates in every cell cycle. The total number of cuts is even larger than the number of effective crossovers later on². The important question of how the sites to be cut are chosen in a given cell—among myriads of potentially equivalent sites that are ignored—is still one of the most vigorously pursued aspects of ongoing research. Foremost, the susceptible substrate for meiotic DSBs is not naked DNA, but DNA embedded in chromatin, as highlighted by Michael Lichten, in his chapter on *Meiotic Chromatin—the Substrate for Recombination Initiation*. The two yeasts compared for this trait show pronounced differences in the distribution of hotspot sites for DSB formation. In *Saccharomyces cerevisiae*, a fairly promiscuous DSB machinery can be assembled at about every stretch of accessible chromatin that has been opened up for other purposes, especially at activated promoter regions. Michael Lichten coins the term "opportunistic DSBs" for these phenomena, foremost in *S. cerevisiae*—differentiating meiotic DSBs from both lower

¹In order of appearance in the text, these actors are known to specialists by acronyms such as Spo11, Top2; Sae2/Com1, Exo1; Rad51, Dmc1; Srs2; RPA; MRX/N; Rad52, Rad54, Mnd1-Hop2, Mei5-Sae3, etc.

²The surplus not leading to crossing-over is eventually repaired from the sister chromatid. Intrinsically, the high value of meiotic recombination can only be compared to recombination accompanying bursts of natural transposon activation characteristic of hybrid dysgenesis syndromes (cf. Gloor and Lankenau 1998). Transposon-encoded transposases/integrases can trigger transposon excision and integration by drastically increasing DSBs and recombination rates between chromosomes—a topic highlighted in the forthcoming book of this Series. Increases in recombination can also result from irradiation-induced DSBs and other genotoxic stress (cf. GDS vol. 1, this SERIES), or during gene targeting experiments, where the free ends that trigger target DNA invasion are brought in from outside the cell.

and higher degrees of sequence specificity: on one hand ionizing radiation-induced DSBs, which occur with little sequence preference and without regard for chromatin structure, and on the other hand from the site-specific cuts of restriction-type endonucleases—or other nucleic acid transactions, such as transcription promotion, where both chromatin structure and the recognition of DNA sequence elements contribute to specificity. Such opportunistic usage of promoter-modulated open chromatin can only in part explain the DSB pattern observed in the fission yeast *Schizosaccharomyces pombe*³, where other determinants may play a significant, hotspot-specific role. Also to be determined by meiosis-specific chromatin organization, the assembly of and/or cleavage by the DSB machinery should not be all too promiscuous on a particular issue, in that at most one of two sister chromatids can become susceptible at any given site, whereas the other sister strand needs to be protected around the equivalent site. The molecular basis for this significant restriction still remains to be determined.

After the meiosis-specific, Spo11-induced DSBs have been processed to protruding 3' ends, these single strands have to interact with the corresponding sequence on the homologous chromosome, in order to repair and seal the break by homologous recombination. In eukaryotes the crucial strand exchange reaction is catalyzed by RecA-like recombinases of the ubiquitous Rad51 family and/or the meiosis-specific Dmc1 protein. As modeled by the most widely studied RecA recombinase of *E. coli*, Chantal Prévost, in her chapter on *Searching for Homology by Filaments of RecA-Like Proteins*, discerns their basic functions in the genome-wide search for complementary DNA strands so as to facilitate the initial strand exchange reaction in highly coordinated, helical DNA-protein filaments, which likewise are formed by the eukaryotic RecA homologs.

Corresponding studies to the leading work on meiosis in *S. cerevisiae* have also been pursued in *S. pombe*, showing striking differences in detail at various levels. The most interesting aspects of this work are pointed out in two chapters specifically devoted to the fission yeast. For one thing, *S. pombe* belongs to the rather few organisms that have lost the ability to form synaptonemal complexes in meiotic prophase, which usually stands out as the most characteristic structural basis of bivalent synapsis. Instead, another conserved feature of canonical meiosis, the clustering of telomeres in the so-called bouquet arrangement, is vastly exaggerated in a series of nuclear movements, which in *S. pombe* facilitates a dynamical alignment of homologous chromosomes from nuclear fusion throughout the entire prophase of meiosis (D.Q. Ding and Y. Hiraoka, this BOOK). Furthermore, the crossover mechanism itself is peculiar as well. Whilst many organisms including *S. cerevisiae* actually employ two partly overlapping crossover pathways, one of these pathways is entirely missing in *S. pombe*. Characteristically, the main recombinational intermediate in *S. pombe* consists of *single* Holliday junctions (G. Cromie and G.R. Smith, this

³The fission yeast *S. pombe* and baker's yeast *S. cerevisiae* are only rather distantly related.

BOOK), whilst earlier results on *S. cerevisiae* had suggested *double* Holliday junctions as the canonical model.

The species-oriented chapter by Gareth Cromie and Gerald R. Smith, on *Meiotic Recombination in S. pombe: A Paradigm for Genetic and Molecular Analysis*, was published Online First in June 2007. At that relatively early date, most of their extensive data on DSB hotspot distribution in *S. pombe* were mentioned in brief as unpublished results. These significant data are now more fully discussed, as mentioned above, in Michael Lichten's comparative chapter—with due reference to their recent publication in the mean time (Cromie et al. 2007). Unfortunate as such asynchrony appears to be, this is a price to pay for the advantages of Online First publication for the individual chapters as they are being completed—with a spread of Online First dates up to a year per book in such a series.

Three evolutionary topics relating to meiosis have been selected to conclude this book: the putative origin of the meiotic system, the confinement of meiosis to the germline in animals, and the abandonment of meiosis in relatively few eukaryotic lineages, some of which are remarkably persistent on the evolutionary time scale—capable of lasting for millions of years. At the dawn of genetics, crossing-over and meiosis had been considered very much the same, but the early view of apparent congruence between the two phenomena has long since been abandoned. Instead, genetic recombination as such has proved to have much earlier and more fundamental roles than the complex and highly integrated pattern of mainstream meiosis, of which crossing-over has become the most characteristic ingredient. In short, homologous DNA recombination has directly co-evolved with faithful replication (see R. Egel and D. Penny, this BOOK), clearing physical damage and/or broken replication forks as they arise (C. Rudolph, K.A. Schürer, and W. Kramer, GDS vol. 1, this SERIES)—potentially in each cell cycle of prokaryotes and eukaryotes alike. Of more sporadic occurrence, on the other hand, *meiosis* only happens once per *generation*, or *life cycle*—what ever meaning may be attached to these derived terms for unicellular organisms (see below). N.B., bacteria and archaea are proficient in recombinational repair of DSB damage to their DNA, but meiosis is missing altogether.

In multicellular organisms, the meanings of *generation* and *life cycle* are evident, and the complex inter-relationship of germline development and maintaining sexuality in animals and plants was already recognized by Charles Darwin and August Weismann by the end of the 19th century. In his chapter on *The Legacy of the Germ Line—Maintaining Sex and Life in Metazoans: Cognitive Roots of the Concept of Hierarchical Selection*, Dirk-Henner Lanke-nau follows the germline concept to its historical roots, and he addresses the multiple levels of selective evolution related to this concept. Also, he fathoms Weismann's prescient usage of *germ plasm* in its original meaning that nowadays has been replaced by *genes* and *genomes*—and he sketches a tie to modern frontiers, discussing the so-called *nuage* as a germline-specific *germ plasm* or-

ganelle of multiple RNA processing, where a suspended term is thus revived in new guises.

A hallmark of meiosis is the production of recombinant offspring, efficiently scrambling the parental genotypes. The overwhelming majority of taxonomic groups throughout eukaryotes show proficiency of meiosis, at least to begin with. Higher plants and animals would probably never have originated without the evolutionary thrust empowered by meiosis. Yet, sexual propagation including meiosis has been lost repeatedly in evolution, although major evolutionary innovations have never sprung from such secondarily asexual lineages. Hence, asexual lineages of relatively ancient origins can serve as virtual mirrors to reflect the evolutionary importance of meiosis in the remaining majority of animals and plants, as thoroughly discussed by Isa Schön, Dunja K. Lamatsch, and Koen Martens in their chapter on *Lessons to Learn from Ancient Asexuals*. To single out a particular highlight, the purging of deleterious mutations by ameiotic recombination appears to be remarkably effective—readily compensating for the low mutation rates observed.

As for the inferred origin of the meiotic system, this does not only far predate the emergence of multicellular animals, fungi and plants—it even dates back before the last common ancestor of all the eukaryotic phyla known today (LECA). As canonical meiosis, therefore, is a common heritage to all eukaryotes, there are no comparative cues among different lineages living today from which by parsimony to deduce a likely order of step-wise additions to the basic toolbox of meiotic mechanisms. On the other hand, the meiotic system is so complex in its widely conserved pattern, that its instantaneous invention from scratch appears unlikely. Against this rather uninformative backdrop, Richard Egel and David Penny, in their chapter *On the Origin of Meiosis in Eukaryotic Evolution*, propose a possible series of incremental steps towards meiosis, each of which could have added some selective advantage on its own. This series may well have started before the mitotic division system had been perfected to its present fidelity, e.g. when telomere-directed chromosome movements may have preceded the establishment of centromeres. Hence their hypothesis is subtitled *Coevolution of Meiosis and Mitosis from Feeble Beginnings*. A likely driving force to establish a proto-meiotic system—alternating with proto-mitotic nuclear division—is seen in maintaining a periodically needed dormancy program, so as to protect it against the accumulation of dormancy-deficient mutations at the higher error load presumed in early evolution. This is in line with the common correlation between meiosis and the formation of dormant spores or cysts in extant microbial eukaryotes. In a certain sense, therefore, a single *generation* in the *life cycle* of unicellular eukaryotes would last from one stage of encystment or sporulation to the next.

With the commissioning and presentation of the various chapter topics on the genomic aspects of the meiotic system we hope to have served a salient need for integrating basic knowledge gained from studying diverse genetic

model organisms. Research on meiotic exchange and segregation mechanisms may appear more esoteric than the vast resources spent on understanding metabolism and growth in mitotic cells. While emphasis on the latter area is motivated by the numerical predominance of mitotic divisions, as well as the direct connection of mitotic cell divisions to the immense problems of cancerous growth in human disease, meiosis in its paucity is more secluded and its medical aspects are limited to less pressing problems, such as impaired fertility or Down-like syndromes (H. Kokotas, M. Grigoriadou, and M.B. Petersen, this SERIES). Also, a certain twist of hierarchy is undeniable: whilst endless perpetuation of mitotic divisions can be viable as an evolutionarily stable strategy, a contiguous series of several meioses is certainly not. In this sense meiosis will always be the subordinate companion of mitosis. At the conceptual level, however, the complexity of molecular mechanisms applying to meiosis far exceeds that of its mitotic counterpart. And for the continuity of generations in most eukaryotic forms of life, both meiosis and mitosis are complementary features of general and essential interest.

Traditionally, the largest share of meiotic research has been focused on DNA exchange and related features, whereas the immense field of protein–protein interactions in the rewiring of the meiotic cell out of and back into the mitotic cell cycle stood in second place. The concluding chapter of the preceding volume specifically deals with these meiotic aspects of molecular cell physiology (L. Pérez-Hidalgo, S. Moreno, and C. Martin-Castellanos, this SERIES). As pioneered with yeasts, genome-wide expression studies have started with identifying all the genes upregulated in meiotic cells and sorting them into functional categories. This is a long way off from knowing all their particular functions. To illustrate the scope of the barely charted field: of 4,824 annotated genes in *S. pombe*, 955 proteins contain *coiled-coil* motifs⁴; of these, 180 are upregulated before, during or after meiosis—21 exclusively so, but not expressed during mitosis (Ohtaka et al. 2007). The interactive potential of so many proteins is enormous, and the *systems biology of meiosis* has merely just begun.

To form a link between both books on *Recombination and Meiosis*, the list of chapter titles in the preceding volume is included after the Contents table of this book. In fact, as some of the individual chapters already had been published Online First, before the editorial decision to divide the printed edition into two books, the preliminary cross references had not yet accounted for the split. We apologize for any inconvenience this may cause, but the listing of all the chapter titles in both books should hopefully direct the reader to the proper destination. We would also like to point out that the missing chapter numbers are no neglect but reflect an obligatory compromise necessitated by publishing all manuscripts OnlineFirst immediately

⁴Coiled-coil motifs often serve as extended dimerization domains, as found in many filament components or structural linker proteins.

after they have been peer-reviewed, revised, accepted and copy-edited (see, <http://www.springerlink.com/content/119766/>).

We most cordially thank all the chapter authors for contributing to this topical edition of two accompanying books focusing on meiotic recombination. Without their expertise and dedicated work this comprehensive treatise would not have been possible. Receiving the incoming drafts as editors, we had the great privilege of being the first to read so many up-to-date reviews on the various aspects of meiotic recombination and model studies elucidating this ever-captivating field. Also, we greatly appreciate the productive input of numerous referees, who have assisted us in thriving for the highest level of expertship, comprehensiveness, and readability.

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Copenhagen,
Ladenburg, April 2008

Richard Egel
Dirk-Henner Lankenau

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Note Added in Proof

In the field of homologous recombination mechanisms, a recent experimental publication stands out as a very important breakthrough paper. Chen, Yang & Pavletich (2008) report crystal structures of RecA microfilaments, comprising five to six interconnected RecA moieties with single-stranded (presynaptic) or heteroduplex (postsynaptic) DNA. The structural coordinates confine the general considerations discussed in Chantal Prévost's chapter to the particular model suggested earlier by Prévost and Takahashi (2003). (i) The RecA-bound presynaptic ssDNA resembles B-form DNA in base-stacked blocks of three nucleotides per RecA subunit, where base stacking is interrupted towards the adjacent triplets. (ii) The ssDNA is bound from the backbone by two flexible

loops L1 and L2 of RecA. The L2 hairpins, in particular, fill in the unstacked space between the adjacent base triplets. (iii) The Watson-Crick edges are freely exposed to the solvent and ready for base pairing with a complementary strand. (iv) Heteroduplex formation with a second strand changes conformation of the primary strand only very little, and the complementary strand is held in position by Watson-Crick base pairing in B-form overall topology, actually with very few protein contacts to RecA. (v) By inference, the stretching-induced disruption of base stacking in the incoming donor duplex likely represents the most important feature in the RecA-mediated strand-exchange reaction.

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Meiotic Chromatin: The Substrate for Recombination Initiation

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Abstract The DNA double-strand breaks (DSBs) that form during meiosis I prophase initiate recombination. DSBs also play a critical role, in many species, in driving progressive association and colocalization of homologs, which culminate in full homolog synapsis at pachytene. Data from many species indicate that DSBs and recombination are not uniformly distributed, but occur more frequently in some places than in others. Studies from *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, where DSBs have been mapped at the molecular level, indicate that chromatin structure is an important determinant of where DSBs form, but that other factors are also involved. Less direct data from other species also address possible roles for chromatin structure and higher-order chromosome structure in DSB formation.

Abbreviations

bp	base pairs
ChIP	chromatin immunoprecipitation
DNase	deoxyribonuclease I
DSB	DNA double-strand break
HS	hypersensitive
IGR	intergenic region
kb	kilobase pairs
mb	megabase pairs
MNase	micrococcal nuclease
ORF	open reading frame
rDNA	ribosomal DNA

1

Introduction

This chapter addresses the question of what determines where meiotic recombination occurs, and in particular the factors that determine where meiotic recombination initiates. In many organisms, recombination drives both homolog pairing and segregation during meiosis (Petronczki et al. 2003; Egel,

this series), and it is therefore critical that recombination occurs in regions where it will lead to productive interhomolog interactions, and avoid adverse consequences.

Although there are many reasons why sites of recombination initiation might be controlled, three are considered here. First, the biochemical steps of recombination initiation by DNA double-strand breaks (DSBs) (Keeney, this series) and subsequent events in interhomolog recombination by strand invasion (Ehmsen and Heyer, this book) involve direct protein–DNA interactions. Most eukaryotic chromosomal DNA is sequestered in nucleosomes, which constitute a barrier to these protein–DNA transactions (Grigoriev and Hsieh 1997; Kwon et al. 2008; Wu and Lichten 1994), and thus DSB formation and strand invasion are expected to occur most readily at places where nucleosomes are either disrupted or absent, and where the underlying chromosomal DNA is accessible. The relationship between chromatin structure and meiotic recombination is a major focus of this chapter.

Second, the use of recombination for the homology search that drives homolog pairing has the collateral consequence of promoting ectopic recombination, recombination between dispersed repeated sequences. This has the potential to disrupt homolog pairing, and can produce deleterious chromosome rearrangements (Haber et al. 1991; Jinks-Robertson and Petes 1985; Kupiec and Petes 1988b; Lichten et al. 1987; Murti et al. 1994; May, Slingsby and Jeffreys, this series). Chromatin modification and gene silencing have potentially important roles in preventing ectopic recombination between repeated sequence elements.

Third, DSB repair and chiasma formation involve the remodeling of both chromatin and chromosome structure (reviewed in Bao and Shen 2007; Kleckner 2006). If recombination occurs in the vicinity of chromosome elements necessary for chromosome segregation (for example, near centromeres), it has the potential to disrupt these structures and thus compromise homolog disjunction (discussed by Koehler et al. 1996; Lamb et al. 1996; Rockmill et al. 2006). This chapter will discuss possible mechanisms that protect chromosomes from the potentially disruptive effects of recombination.

Although the control of meiotic recombination patterns has been the focus of intense study in a variety of eukaryotic organisms, knowledge in the field remains fragmented and idiosyncratic. Only in recent years have whole-genome (or even whole-chromosome) mapping studies provided a picture of where meiotic recombination actually occurs, and efforts to relate this picture to underlying chromatin and chromosome structure have only just begun. Not surprisingly, studies in different species tend to focus on aspects of the problem that are experimentally tractable, and thus there is no single system in which a comprehensive picture can be drawn from underlying chromatin/chromosome structure to recombination initiation and then to the final outcome of meiotic recombination. For example, detailed whole-genome DSB maps are available in *Saccharomyces cerevisiae* (Blitzblau et al. 2007;

Buhler et al. 2007; Gerton et al. 2000) and whole-genome chromatin structure mapping efforts are under way (Rando 2007), but high-resolution recombination maps are lacking. By contrast, detailed crossover maps are available in *Homo sapiens* and other mammalian species (reviewed in May, Slingsby and Jeffreys, this series), but the distribution of the molecular events that lead to these crossovers and their relationship with underlying chromatin structure remains unexplored.

As a consequence, it is currently not possible to draw comprehensive conclusions regarding the relationship between chromatin/chromosome structure, chromatin modification, and meiotic recombination. Instead, this chapter briefly summarizes what is known from studies in different species, concentrating on *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, for which the greatest detail is known. Further information can be gained from recent review articles (Buard and de Massy 2007; Coop and Przeworski 2007; de Massy 2003; Jeffreys et al. 2004; Kauppi et al. 2004; Keeney and Neale 2006; Kleckner 2006; Koren et al. 2002; Mézard 2006; Topp and Dawe 2006) and from several chapters in this series (Keeney; Ashley; May, Slingsby and Jeffreys; Jones and Franklin; this series, Cromie and Smith; this book).

2

Double-Strand Breaks and Chromatin Structure in *Saccharomyces cerevisiae*

Meiosis-induced DSBs were first discovered in *S. cerevisiae* and have been most extensively characterized in this organism (Keeney, this series). DSB mapping efforts have made extensive use of mutants (*rad50S*, *sae2* and *mre11S*, referred to here as *rad50S*-like) where DSBs form but are not further processed, and Spo11, the enzyme that forms breaks, remains linked to DSB ends (Cao et al. 1990; Keeney and Kleckner 1995; McKee and Kleckner 1997; Nairz and Klein 1997; Prinz et al. 1997). These mutants accumulate unprocessed DSBs that can be mapped at high resolution on Southern blots (de Massy et al. 1995; Liu et al. 1995; Xu and Petes 1996; Xu and Kleckner 1995), and that can be used to detect break-adjacent sequences by immunoprecipitation of the covalently linked Spo11 moiety (Borde et al. 2004; Gerton et al. 2000; Keeney et al. 1997; Prieler et al. 2005). This has allowed direct comparisons between DSB patterns and features of chromatin structure.

2.1

DSBs Form in Open Chromatin

Budding yeast lacks what is classically considered heterochromatin, and heterochromatin-associated histone modifications, histone-modifying complexes, and heterochromatin-binding proteins are absent. Instead, where regional transcriptional repression occurs (at telomeres, silent mating-type cas-

settes, and the rDNA), it is accomplished by histone deacetylation and direct recruitment of specialized silencing proteins (Rusche et al. 2003). The bulk of the yeast genome resides in open chromatin, characterized by the following stereotypical pattern. A typical yeast gene (Fig. 1A) contains a 5' promoter region where nucleosomes are absent or disrupted, and this open region is flanked by nucleosomes that contain the histone H2A variant, H2Az (Liu et al. 2005; Raisner et al. 2005; Yuan et al. 2005). Coding and 3' downstream sequences are nucleosome-occupied in all but the most highly transcribed genes. Open sites in promoter chromatin, where the underlying DNA is readily available for binding and modification by non-histone proteins, are detected as nuclease-hypersensitive (HS) sites in deoxyribonuclease I (DNase I) or micrococcal nuclease (MNase) digests of chromatin (Gross and Garrard 1988).

Studies of several loci identified gradients of meiotic gene conversion where the high end of the gradient was at the 5' end, consistent with the presence of a recombination initiator in the promoter (Detloff et al. 1992; Fogel et al. 1981; Nicolas et al. 1989; Schultes and Szostak 1990; Vedel and Nicolas 1999). Both *cis*-acting promoter deletions and mutations in *trans*-acting transcription factors abolish or reduce conversion gradients (Detloff et al. 1992; Nicolas et al. 1989; Schultes and Szostak 1990, 1991; White et al. 1991, 1993), suggesting that features of promoter region structure or function contribute to initiator activity. This was supported by the discovery that DSBs frequently form during meiosis in the same promoters that contain gene conversion hotspots (Fan et al. 1995; Goldway et al. 1993; Sun et al. 1989), and preferential DSB formation in promoter regions was also documented in studies of larger regions (Wu and Lichten 1994) and of the entire length of chromosome *III* (Baudat and Nicolas 1997). The frequent localization of DSBs in promoter regions, known to be more open than bulk chromatin, suggested a correlation between sites of DSB formation and open sites in chromatin.

This correlation has been confirmed at a number of DSB hotspots. In most cases, DSBs occur in restricted regions, on the order of 50–250 bp (de Massy et al. 1995; Liu et al. 1995; Xu and Petes 1996; Xu and Kleckner 1995) that are nuclease HS in digests of chromatin (Borde et al. 1999; Fan and Petes 1996; Keeney and Kleckner 1996; Kirkpatrick et al. 1999; Wu and Lichten 1994, 1995). However, DSBs can occur over larger regions that are nucleosome-free. This is best illustrated at the *PHO5* promoter, which is occupied by positioned nucleosomes when transcription is repressed and is nucleosome-free over a ~500-bp region when transcription is activated (Almer et al. 1986). DSBs at *PHO5* correlate precisely with promoter chromatin structure (Wu and Lichten 1994; Fig. 1B), with the pattern of DSBs changing from a single DSB site, in the repressed state, to DSBs being distributed over the entire nucleosome-free region created upon induction. Deletion mutants that remove the *PHO5* TATAA box and that reduce transcription tenfold (Fascher et al. 1993) still undergo both nucleosome removal and DSB region expansion

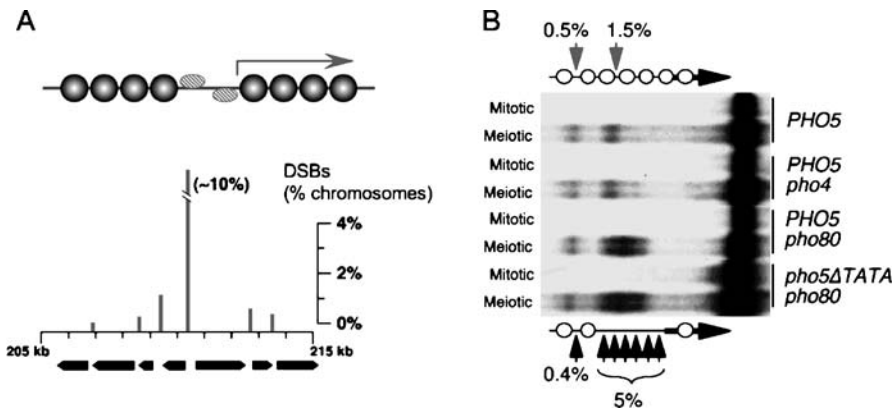


Fig. 1 Chromatin structure determines where meiosis-induced DSBs occur in *S. cerevisiae*. **A** *Top*: Cartoon of chromatin structure in a typical yeast gene. Transcription factors and underlying DNA sequence combine to exclude nucleosomes from the promoter region of a gene, while surrounding regions, including coding sequences, are occupied by positioned nucleosomes. *Bottom*: DSBs occur primarily in transcription promoters. Map of DSBs and protein-coding genes in the vicinity of *ARE1* on chromosome III, showing coding sequences (horizontal arrows), genes and direction of transcription; vertical bars, DSB locations and intensities). *ARE1* is the large open reading frame (ORF) immediately to the right of the most prominent DSB site. Modified from (Wu and Lichten 1994).

B DSBs in the *PHO5* promoter (Wu and Lichten, unpublished data). Induction of *PHO5* expression is accompanied by the removal of positioned nucleosomes from the *PHO5* promoter (repressed state illustrated above Southern blot; induced state illustrated below Southern blot). Both wild-type cells and *pho4* mutants are repressed, while *pho80* mutants are constitutively induced during meiosis. This figure contains a Southern blot of DNA from *rad50S* strains with the indicated genotypes, probed for DSBs in the *PHO5* promoter region. *PHO5* induction is accompanied by a substantial increase in DSBs, which occupy much of the region of nucleosome depletion; this occurs even when transcription is reduced tenfold by a TATAA-box deletion (*pho5ΔTATA*). DSB frequencies are indicated in terms of percent of total DNA

(Fig. 1B), consistent with the conclusion that chromatin structure, rather than transcriptional activity, determines where DSBs form (White et al. 1992). This conclusion is further supported by the detection of DSB hotspots in nuclease-HS regions that are fortuitously created by the juxtaposition of yeast and bacterial sequences in artificial inserts into yeast chromosomes (Keeney and Kleckner 1996; Wu and Lichten 1995; Xu and Petes 1996).

To summarize, current data suggest that chromatin structure is the most basic determinant of where meiotic recombination initiates in *S. cerevisiae*. DSBs form where underlying DNA is exposed, and thus is available for binding by Spo11 and associated proteins (see Keeney, this series). Thus, at the most basic level, meiotic recombination initiation in *S. cerevisiae* is opportunistic. Rather than remodeling chromatin at predetermined sites to create a substrate for cleavage, the DSB-forming machinery makes use of a preexist-

ing substrate, open chromatin, which is established for other purposes, most commonly for promoting transcription.

This conclusion begs the question of what creates open chromatin in the first place, and whether or not the factors that create open chromatin are also involved in directly recruiting DSB-forming proteins. While DNA sequences that directly exclude nucleosomes can underlie both nuclease-HS sites and DSB hotspots (Kirkpatrick et al. 1999), many DSB hotspots are located in open chromatin that is created when transcription factors bind and recruit chromatin-modifying and chromatin-remodeling complexes. Studies of the recombination hotspot in the *HIS4* promoter show that the binding of transcription factors with intact activation domains, but not transcription per se, is required for hotspot activity (reviewed by Petes 2001). Deleting transcription factor binding sites in the *HIS4* promoter, or mutating genes encoding the transcription factors that bind to those sites, decreases meiotic recombination and DSB formation, while deleting the *HIS4* TATAA box has no effect on recombination (Fan et al. 1995; White et al. 1991, 1992, 1993). It remains to be seen if hotspot activity at *HIS4* results simply from changes in chromatin structure at the target site, or if transcription factors that bind to the *HIS4* promoter play a role in directly recruiting the DSB-forming protein complex.

2.2

Chromatin Structure and Postinitiation Events

DSB formation in preexisting open sites in chromatin has the additional advantage of facilitating later steps of meiotic recombination (see Ehmsen and Heyer, this book). DSBs that form in open chromatin are, of course, usually repaired using allelic sequences that are also in open chromatin, reducing the need for chromatin remodeling during strand invasion. This may also facilitate initial homology searching, if this process is more efficient with naked DNA than with DNA that is partially occluded by nucleosomes. Since a substantial majority of yeast sequences are nucleosome-occupied (Yuan et al. 2005), factors that focus DSB formation in open chromatin may substantially reduce the total sequence space that must be searched during recombination-driven homolog pairing.

DSB targeting to open chromatin also helps to control recombination between endogenous repeated elements. Ty elements are a dispersed family of 30–40 retrotransposons that represent about 3% of the yeast genome (Kim et al. 1998). While ectopic meiotic recombination occurs frequently between dispersed artificial repeats that are similar in size to Ty elements (Lichten et al. 1987), meiotic exchange between Ty elements is much less frequent (Kupiec and Petes 1988a,b). Ty elements are actively transcribed in haploid cells but are repressed in cells (such as diploids) that express both *MAT* locus alleles (Company and Errede 1988). Studies of a Ty element inserted upstream of *HIS4* showed that this repressed state is associated with a compact chromatin

structure, with an absence of DNase-HS sites (and DSBs) in both the Ty element and flanking sequences (Ben-Aroya et al. 2004). Thus, ectopic meiotic recombination between Ty elements is most likely prevented by transcription repression mechanisms, which create a chromatin structure that is incompatible with meiotic DSB formation in cells with the potential to undergo meiosis.

Chromatin structure and modification status may also limit meiotic recombination in the ribosomal RNA gene cluster on chromosome *XII*, which is normally a recombination desert (Petes and Botstein 1977). Both recombination and DSB formation within and near the rDNA repeats are increased in mutants lacking the Sir2 histone deacetylase (Gottlieb and Esposito 1989; Mieczkowski et al. 2007). Since Sir2 is required for chromatin compaction and repression of Pol II-promoted transcription at sites in rDNA (Fritze et al. 1997), increased rDNA meiotic recombination in *sir2* mutants may be a consequence of increased chromatin accessibility and DSB formation at these sites, but this remains to be tested experimentally.

2.3

A Meiotic Chromatin Transition at Active DSB Sites

Several DSB sites, most notably those in the *ARG4* and *CYS3* promoters, undergo a change in chromatin structure that occurs before DSB formation (Ohta et al. 1994). This change is revealed as a quantitative increase in MNase sensitivity at the nucleosome-free region where DSBs occur. Studies by Ohta and coworkers have shown that this chromatin structure change is intimately related to DSB formation. The extent of chromatin opening parallels DSB frequencies in *ARG4* promoter deletion mutants that increase or decrease recombination (Ohta et al. 1994). Furthermore, when the *ARG4* DSB site is inactivated by competition with nearby DSB hotspots (see Sect. 2.4), the chromatin transition at *ARG4* is also eliminated, while nearby active DSB sites retain a meiosis-specific increase in MNase sensitivity (Ohta et al. 1999). Finally, in strains where DSB formation in the *ARG4* promoter is ~ 1 h later than is normal, the chromatin transition is similarly delayed (Murakami et al. 2003).

The molecular nature of this chromatin transition remains undetermined. It is unlikely that it represents substantial changes in nucleosome occupancy and/or location, as sites that show increased MNase sensitivity do not show similar sensitivity increases in chromatin digests with DNase I, which is less sensitive to non-histone protein occupancy than is MNase and shows different DNA structural preferences (Wu and Lichten, unpublished; Keeney, personal communication). Increased MNase sensitivity might reflect changes in histone modification or non-histone protein occupancy that reflect changes in promoter function; this seems unlikely, given that a similar meiotic increase in MNase sensitivity is seen at DSB sites located in plasmid sequences

that are inserted in the yeast genome, and which are unlikely to contain transcription promoters (Ohta et al. 1999). It has been suggested that increased MNase sensitivity reflects the binding of DSB-forming proteins, which has been shown to occur before break formation (Prieler et al. 2005; Sasanuma et al. 2008). The latter suggestion is supported by two findings. First, the chromatin transition does not occur in mutants lacking Mre11 (Ohta et al. 1998), which is required for break formation and which is recruited to DSB sites in a manner that is independent of break formation (Borde et al. 2004). Second, the same meiotic chromatin transition occurs at the *GAL2* promoter in cells constitutively expressing a fusion protein containing the Gal4 DNA-binding domain and Spo11 (Gal4BD-Spo11) (Pecina et al. 2002). Because GalBD-Spo11 binds the *GAL2* promoter constitutively (Sasanuma et al. 2007), this chromatin transition most likely reflects the Spo11-dependent recruitment of other proteins. It remains to be determined if these are the DSB-forming machinery itself, or if they are proteins, recruited by GalBD-Spo11, that modify chromatin in advance of DSB formation.

2.4

Other Factors that Influence DSB Patterns

While all DSB sites examined to date are nuclease-HS sites in chromatin, not all nuclease-HS sites are efficiently used as meiotic DSB sites (reviewed in Petes 2001; Keeney, this series). This suggests that factors other than chromatin structure help to determine where recombination initiates in the budding yeast genome. Studies of regional, chromosomal, and whole-genome DSB patterns have identified some of these factors, although mechanistic insight is still forthcoming.

Interpretation of many of these studies is complicated by the fact that most studies have mapped and quantified DSBs in *rad50S*-like mutants, which allow DSB formation but block the removal of covalently linked Spo11 from DSB ends (Keeney and Kleckner 1995; Neale et al. 2005; reviewed by Keeney, this series). It is now clear that DSB patterns in these mutants do not accurately reflect DSB and recombination patterns that occur in wild type. In particular, *rad50S*-like mutants display alternating 50–200-kb domains of DSB enrichment alternating with similarly sized domains of DSB depletion (Baudat and Nicolas 1997; Borde et al. 2004; Gerton et al. 2000). This is not observed when Spo11 removal and DSB processing is permitted (Blitzblau et al. 2007; Borde et al. 2000; Buhler et al. 2007; Dresser et al. 1997). Instead, a more even DSB distribution is observed, with the vast majority of regions showing substantial DSB activity (Fig. 2). The time of DSB formation is one factor responsible for this phenomenon, as later-than-average DSB formation is associated with regional DSB underrepresentation in *rad50S*-like mutants (Borde et al. 2000). However, it is unlikely that DSB timing is the only factor responsible. For example, at some DSB sites that are underrepresented in

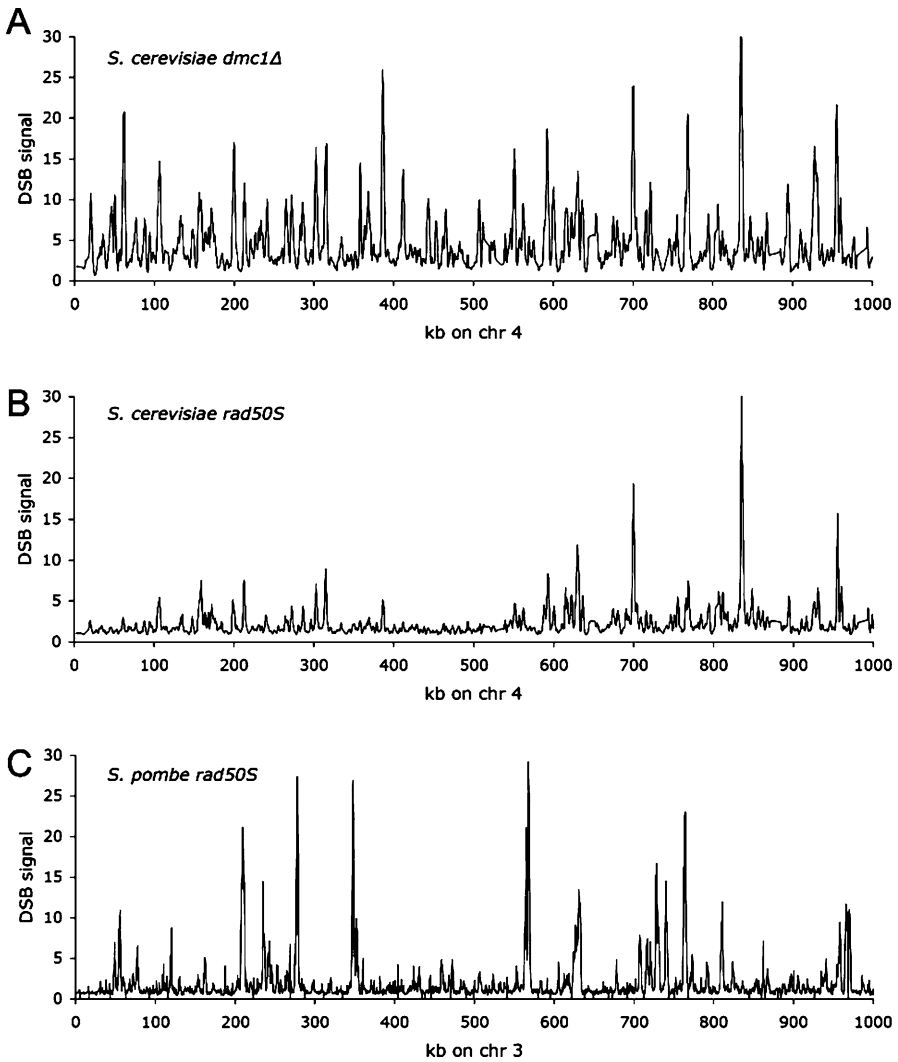


Fig. 2 DSB patterns in *S. cerevisiae* and *S. pombe*. This figure contains plots of DSB signals from whole-genome microarray studies. DSB signals are from the first 1000 kb of chromosome 4 of *S. cerevisiae* and from the first 1000 kb of non-rDNA sequences on chromosome 3 of *S. pombe*. Data (Buhler et al. 2007; Cromie et al. 2007) are reproduced and reanalyzed under a Creative Commons License. **A** DSB signals from an *S. cerevisiae dmc1Δ* mutant, detected using single-strand DNA enrichment, showing a typical pattern of DSB hotspots that are broadly and relatively uniformly distributed. **B** DSB signals from an *S. cerevisiae rad50S* mutant, detected using Spo11-ChIP, show a typical pattern of DSB hotspot clusters separated by regions where there are few DSBs. **C** DSB signals from an *S. pombe rad50S* mutant, detected by Rec12-ChIP (Rec12 is the *S. pombe* Spo11 homolog), showing a typical pattern of intense DSB hotspots separated by large (> 50 kb) regions where there are few DSBs

rad50S-like mutants, breaks form at the same time as DSBs at other loci that show wild-type DSB levels in *rad50S* (Buhler et al. 2007).

Despite this potential complication, it is clear from a variety of studies that higher-order chromosome structure affects DSB patterns. In particular, DSBs are significantly underrepresented in the vicinity of both telomeres and centromeres, with DSB underrepresentation occurring in about 10–20 kb around centromeres and about 20 kb next to telomeres (Blitzblau et al. 2007; Buhler et al. 2007). Although part of this effect can be ascribed to reduced gene density (and thus reduced promoter density) near these elements, it must also be due to direct DSB repression. When recombination-reporter plasmids that contain active DSB sites are inserted near centromeres and telomeres, they show reduced DSB and recombination activity, but unaltered DNase-HS patterns (Borde et al. 1999; Robine et al. 2007; Wu and Lichten, unpublished).

Centromere translocation studies of chromosome *III* have shown that recombination and DSB repression in pericentric sequences are caused by the centromere itself. Centromere deletion causes substantial increases in DSB formation and recombination in the former pericentric region, and the insertion of *CEN3* at other locations causes substantial DSB and recombination decreases near the new centromere (Lambie and Roeder 1986, 1988; Robine et al. 2007; Wu and Lichten, unpublished data). Several structural features of pericentric regions might contribute to DSB repression. Cohesin binds preferentially in a ~20-kb pericentromeric domain similar in size to the region of DSB repression (Blat and Kleckner 1999; Glynn et al. 2004), and other preferential cohesin binding sites on chromosome arms are negatively correlated with DSB hotspots. The Zip1 protein, a component of the central element of the synaptonemal complex (see Suja and Rufas, this series), is transiently bound to centromere regions in early meiosis I prophase (Tsubouchi and Roeder 2005). Either cohesin or Zip1 complexes might create a structural environment that is refractory to DSB formation. Alternatively, other aspects of pericentric chromosome structure may block DSB formation. Bloom and coworkers recently have proposed that kinetochores adopt a cruciform structure, in which chromatids are organized into ~20-kb hairpins of intrachromatid cohesion and sister chromatid separation (Yeh et al. 2008). A requirement for sister chromatid association could thus account for observed DSB reductions in pericentric regions; however, it should be noted that DSBs do form in mutants lacking Rec8, the meiosis-specific kleisin subunit of cohesin (Klein et al. 1999). It also should be noted that the Red1 axial element protein, which is required for efficient DSB formation, is underrepresented in the cohesin-rich pericentric region of chromosome *III* (Blat et al. 2002; see discussion at the end of this section).

DSB repression near telomeres is less well studied, but it is intriguing that the region affected (~20 kb) is about the same size as the region of Sir2-mediated transcription repression seen adjacent to telomeres (Rusche et al. 2003). *sir2* mutants display increased DSBs in the 10–20 kb immediately ad-

jacent to telomeres and in the rDNA and flanking sequences, which are also subject to Sir2-mediated repression (Mieczkowski et al. 2007). These results can be understood as reflecting increased histone acetylation, and thus increased chromatin accessibility, in regions that are normally repressed by Sir2-mediated histone deacetylation. However, *sir2* mutants also show an apparent DSB decrease in a broad region near but not adjacent to telomeres (on the average, 20–150 kb from telomeres). This cannot be readily explained by known changes in chromatin structure or modification, and raises the possibility that larger-scale changes in chromosome and/or nuclear structure or dynamics may be responsible for the altered DSB patterns seen in *sir2* mutants.

In addition to histone acetylation, histone methylation and ubiquitylation have also been suggested as being important for DSB formation. Mutants lacking Rad6 or Bre1, both involved in the ubiquitylation of histone H2B at lysine 123, show reduced DSBs, as do mutants lacking the target lysine. DSBs formed at Gal4 binding sites by a Gal4BD–Spo11 fusion are not affected, suggesting that H2B ubiquitylation is involved in recruiting DSB-forming protein complexes to native DSB sites (Yamashita et al. 2004). The Set1 methyltransferase, which methylates histone H3 at lysine 4 in transcribed genes, has also been implicated in DSB formation (Sollier et al. 2004). Profound DSB defects are seen in *set1* mutants, but these mutants also show delayed premeiotic replication and altered meiotic transcription. These pleiotropic effects, which are typical of mutants that alter global histone modification, make it difficult to distinguish direct effects on DSB formation from indirect effects that might involve altered expression of one or more genes necessary for DSB formation. The latter is certainly true in the case of Gcn5, a histone H3 acetylase. *gcn5* mutants show profound defects in DSB formation, but this can be ascribed to a failure to express *IME2*, a kinase that is necessary for early events in meiosis I prophase, including DSB formation (Burgess et al. 1999).

DSB formation also is influenced by the presence or absence of other DSB sites, as insertion or creation of strong DSB hotspots causes a general suppression, in *cis*, of DSBs at nearby sites (Fan et al. 1997; Jessop et al. 2005; Robine et al. 2007; Wu and Lichten 1995; Xu and Kleckner 1995). In two cases, sites where DSBs are suppressed are as open (as measured by DNase I sensitivity) as the same sites when DSBs are active (Borde et al. 1999; Wu and Lichten 1995), although the competitively suppressed sites do not display the increase in MNase digestion that occurs at active DSB sites (Ohta et al. 1999). DSB suppression can extend over remarkably long distances, up to 60 kb from the strong DSB hotspot (Jessop et al. 2005; Robine et al. 2007), and operates primarily in *cis* (Wu and Lichten, unpublished). The mechanisms underlying DSB suppression are not understood, but DSB suppression may reflect a local limitation of factors necessary for DSB formation that are not freely diffusible. Alternatively, DSB suppression could result from DSB-induced regional changes in chromatin modification and/or chromatin

compaction (reviewed by Downs et al. 2007) that might inhibit DSB formation at second sites.

Finally, phenotypes of mutants lacking lateral element proteins (Hop1 and Red1 in budding yeast; see Suja and Rufas, and Keeney, this series) suggest a role for higher-order meiotic chromosome structure in determining DSB distributions. Meiotic chromosomes are organized into large loops (10–20 kb in budding yeast), with sequences at the base of loops associated with the chromosome axis and lateral elements (Blat et al. 2002). DSBs form in loop sequences (Blat et al. 2002) but, somewhat paradoxically, are substantially reduced in *hop1* and *red1* mutants (Keeney 2001). In addition, cytological studies in several organisms indicate that the Rad51 and Dmc1 strand transfer proteins, which bind to DSB-linked single-strand DNA, are found in foci that are located on lateral elements (Moens et al. 2002; Ashley, this series). This apparent paradox can be explained if DSB-forming proteins initially bind to sites on chromosome loops, and these protein–DNA complexes need to move to the chromosome axis to trigger DSB formation (Blat et al. 2002; van Heemst and Heyting 2000; see discussion in Keeney, this series). If target sites on lateral elements are limited, and if different loop sites encounter lateral elements to differing extents, differences in DSB frequencies at sites with similarly open chromatin could be accounted for, as could the phenomenon of competitive DSB suppression discussed above.

2.5

Areas for Future Study

While it is now clear that features of chromatin structure, in particular nucleosome-free regions, serve as the underlying substrate for DSB formation in *S. cerevisiae*, it is also clear that this cannot entirely account for all features of DSB patterns in the budding yeast genome. Much should be learned if methods recently used for genome-wide DSB mapping are extended to the analysis of chromatin dynamics and modification, and to the location and dynamics of proteins involved in DSB formation. Studies of competitive DSB suppression and of DSB activity in reporter inserts indicate that DSB formation in budding yeast is controlled, in part, at the regional level. Progress in this area will require the refinement and application of tools to examine higher-order yeast chromosome structure.

Finally, it is somewhat incongruous that, given the initial development of *S. cerevisiae* as an organism for genetic study, so much more is currently known about genome-wide DSB patterns than is known about the genome-wide distribution of the recombination events that follow. If progress is to be made in understanding the impact of chromosome structure on the later steps of meiotic recombination, initial efforts at the genome-wide mapping of meiotic exchange in yeast (Winzeler et al. 1998) will need to be extended and refined.

3

Recombination Hotspots and Chromatin Structure in *Schizosaccharomyces pombe*

The relationship between meiotic recombination and chromatin/chromosome structure in *S. pombe* has also been the subject of systematic study, and the findings obtained in this organism provide an interesting contrast to those obtained from budding yeast. Meiotic recombination in *S. pombe* is extensively discussed elsewhere in this volume (Cromie and Smith, this book), and the reader is encouraged to consult that chapter for details not presented here.

Until recently, recombination hotspot characterization in *S. pombe* has focused on hotspots related to *ade6-M26*, a mutation-associated recombination hotspot (Gutz 1971) that creates a binding site for an ATF/CREB transcription factor (Kon et al. 1997). Considerable attention has been paid to chromatin structure changes associated with recombination activity at sites that contain the *M26* sequence motif, but recent studies indicate that other types of hotspots are more prevalent (Cromie et al. 2007; Steiner and Smith 2005). Characterization of chromatin structure and dynamics at these elements is just beginning. *S. pombe* also contains heterochromatin similar in composition and character to that found in higher eukaryotes, and thus provides a model system to study the effect of heterochromatin on meiotic recombination. This section will discuss all three of these topics.

3.1

***M26*, a Transcription Factor-Associated Recombination Hotspot**

Mutation analysis of the *ade6-M26* recombination hotspot identified a seven-base sequence necessary for hotspot activity (Schuchert et al. 1991), which is also active as a recombination hotspot when created by directed mutagenesis at other sites in the *ade6* and *ura4* genes (Fox et al. 1997). *M26*-dependent DSB formation has been documented for several of the *ade6* mutations, with DSBs being distributed in a 0.5–1 kb region around the *M26* motif, and DSBs also form in the *ade6* promoter (Steiner et al. 2002). *M26* is contained in a larger sequence motif that is associated with DSBs at 10/15 places in the *S. pombe* genome (Steiner and Smith 2005). However, five of these *M26*-containing sites are apparently inactive, *M26* is inactive on a multicopy plasmid and on certain plasmid inserts at *ura4* (Ponticelli and Smith 1992; Virgin et al. 1995), and an *ade6* promoter deletion abrogates *ade6-M26* hotspot activity in *cis* (Zahn-Zabal et al. 1995). Thus, it appears likely that chromatin and/or chromosome context play an important role in *M26* activity, but the nature of these “outside influences” remains to be determined.

M26 is part of a binding site for the Atf1/Pcr1 heterodimer, a cAMP-dependent stress-response transcription factor (Kon et al. 1997; Wahls and

Smith 1994). Atf1 and Pcr1 are both required for hotspot activity, as are the protein kinases and other proteins in the signaling pathway that activates Atf1/Pcr1 (Fox et al. 2000; Mizuno et al. 2001). Like most transcription factors, Atf1/Pcr1 acts by recruiting histone modifiers and remodelers to open chromatin in promoter regions, and this is closely associated with *M26* hotspot activity.

MNase digests of chromatin show that *M26* alters chromatin structure over a large (~ 0.7 kb) region (Mizuno et al. 1997). Normal *ade6* chromatin contains an MNase-HS site in the promoter and positioned nucleosomes over coding sequences, and displays a modest increase in MNase sensitivity at the promoter site in meiosis. In contrast, *ade6-M26* disrupts nucleosome positioning over much of the *ade6* ORF but does not increase MNase sensitivity during mitosis, suggesting that the ORF is still nucleosome-occupied. During meiosis, there is a strong induction of MNase sensitivity, both at sites in the promoter and at the *M26* consensus sequence (Mizuno et al. 1997), and this chromatin transition requires Atf1/Pcr1 and the associated upstream signaling cascade (Mizuno et al. 2001; Yamada et al. 2004). Chromatin opening at *ade6-M26* is prevented during mitosis by two *S. pombe* homologs of the *S. cerevisiae* Tup1 transcriptional repressor (Hirota et al. 2003), and by the Hrp1 CTD-1 family chromatin remodeler (Hirota et al. 2008). Meiotic induction of MNase-HS at *M26* is associated with hyperacetylation of histones H3 and H4 in surrounding sequences (Yamada et al. 2004), and the *M26*-associated meiotic chromatin transition (and hotspot activity) requires the SAGA histone acetylase complex and the SWI/SNF family and Hrp3 CHD-1 family chromatin remodelers (Hirota et al. 2008; Yamada et al. 2004). The chromatin transition does not require the DSB-forming machinery, as it occurs in cells lacking Rec12, the *S. pombe* Spo11 homolog (Hirota et al. 2008). Interestingly, a recent study showed that *ade6-M26* also causes some *ade6* transcription start sites to shift from the normal promoter to a site just 3' of the *M26* mutation, further supporting the suggestion that *M26* induces a functional change in chromatin structure (Hirota et al. 2008).

In summary, current data are consistent with the suggestion that *M26* recombination hotspot activity results from the binding and activation of the Atf1/Pcr1 heterodimer, which in turn recruits chromatin modifiers and remodelers that create a promoter-like open site in chromatin where DSBs can form. It is unlikely, however, that all features of *ade6-M26* are typical of all *S. pombe* DSB/recombination hotspots, or even of hotspots that contain an *M26* element. Two other *M26*-containing DSB hotspots, one at the *tdh1*⁺ locus and the other at the *cds1*⁺ locus, have recently been examined (Hirota et al. 2007). Both are DSB sites, with the *tdh1*⁺ hotspot undergoing an Atf1/Pcr1-dependent meiotic chromatin transition. MNase-HS at the *cds1*⁺ hotspot is Atf1/Pcr1-dependent but is present in both mitotic and meiotic cells.

3.2

Other Recombination/DSB Hotspots

While *M26*-like elements have been a major focus of investigation, most meiotic recombination in *S. pombe* is Atf1/Pcr1-independent (Kon et al. 1997; Steiner and Smith 2005), which indicates that there are other ways to make a DSB/recombination hotspot. Recombination hotspots can be created by integrating *ura4*- or *ade6*-containing constructs at other loci (Virgin et al. 1995; Zahn-Zabal et al. 1995), but these new hotspots have not been characterized at the molecular or chromatin levels. More recently, genome-wide DSB studies using *rad50S* mutants have revealed the widespread existence of DSB sites that are not easily explained by invoking promoter-associated open chromatin (Cromie et al. 2007). Eighty-eight percent of DSBs occur at 194 sites, with an average spacing of ~ 65 kb between sites (see Fig. 2 for an example). About two-thirds of these sites are located in large intergenic regions (IGRs) of 2 kb or greater; these regions are considerably larger than IGRs in the genome as a whole (median intergenic size of 0.7 kb). The best-characterized large IGR-associated hotspot, called *mbs1*, contains several DSB sites in a 2-kb region (Cromie et al. 2005). These DSB sites are MNase-HS, but they are constitutively open in both mitosis and meiosis, and both DSB formation and chromatin opening are Atf1/Pcr1-independent (Hirota et al. 2007). It remains to be determined whether or not DSB sites in large IGRs are located in transcription promoters. Since few *S. pombe* promoters have been characterized in detail, it is not clear if these large IGRs simply represent very large promoter regions, or if they contain other uncharacterized chromosome structural elements.

In summary, data from *S. pombe rad50S* mutants indicate that, while DSBs can form in transcription promoter sequences near protein-coding genes, most DSBs form elsewhere. If these data reflect genome-wide patterns of DSBs in wild type (as has been shown for one region by Cromie et al. 2007), it would seem that only a fraction of potential DSB sites are used, and other features of chromatin or chromosome structure are required. While the presence of additional proteins that recruit the DSB-forming machinery to specific loci remains a distinct possibility, it is also possible that aspects of higher-order chromosome structure are required. In this regard, it may be instructive to compare *S. cerevisiae* replication origins, which are located in relatively small IGRs (mean size ~ 0.85 kb) and involve the recognition of short sequence motifs (Nieduszynski et al. 2006), to *S. pombe* replication origins, which involve sequence elements distributed over > 1 kb and are preferentially located in large IGRs (Dai et al. 2005; Hayashi et al. 2007; Heichinger et al. 2006). Cromie et al. did not observe an association between DSB hotspots and replication origins (Cromie et al. 2007), making a direct connection unlikely. However, the preferential localization of DSB hotspots in large IGRs, and the distribution of DSBs over kb-size domains within these hotspots, raises the

intriguing possibility that target recognition by *S. pombe* DSB-forming proteins involves a distributed sequence recognition mechanism similar to that used in *S. pombe* replication origins. It is also possible that the preferential location of DSB hotspots in large IGRs reflects features of higher-order chromosome structure and dynamics, such as increased interaction with the chromosome axis (see discussion in Sect. 2.5; also in Cromie and Smith, this book, and Keeney, this series).

As is seen in *S. cerevisiae*, *S. pombe* DSB hotspots display competitive DSB suppression. Deletion of the prominent DSB site *mbs1* greatly decreases DSB frequencies at that site and increases DSBs at sites about 100 kb to each side of *mbs1* by about twofold (Hyppa and Smith, personal communication). Competitive suppression may contribute to the focusing of DSB activity in a small number of widely spaced, intense DSB hotspots, while causing further depletion of DSBs at potential sites in intervening chromosomal sequences.

3.3

Recombination Repression by Heterochromatin

Fission yeast, like most eukaryotes, contains large portions of the genome packaged in heterochromatin, which is characterized by a compact chromatin structure, methylation of histone H3 lysine 9, and the general repression of RNA polymerase II-dependent promoters in genes that are inserted within these regions (reviewed in Grewal and Elgin 2007; Grewal and Jia 2007). The heterochromatic state is established and maintained through the combined and interdependent activity of histone deacetylases, histone methyltransferases, histone-binding proteins, and the RNA-interference (RNAi) machinery, as well as by RNA polymerase II transcription of repeated sequences to support RNAi. In *S. pombe*, heterochromatin is found at subtelomeric and pericentric repeat sequences, and also in the 20-kb interval between the two silent mating-type genes *mat2* and *mat3*. Meiotic recombination is greatly reduced relative to the genome-wide average across *S. pombe* centromeres and pericentric repeats (Nakaseko et al. 1986), and meiotic DSBs are not found in these regions (Cromie et al. 2007). Meiotic recombination is also greatly reduced across the silent mating-type interval (Egel 1984), and mutants lacking heterochromatin-associated proteins simultaneously disrupt heterochromatic silencing and restore meiotic recombination in this region (Grewal et al. 1998; Hall et al. 2002; Thon and Klar 1992). While it remains to be shown that DSBs are absent from the silent mating-type interval during diploid meiosis, DSBs are absent from this region in *pat1-ts* haploid cells that are induced to undergo meiosis by a shift to the nonpermissive temperature (Cromie et al. 2007).

Although it is attractive to suggest that heterochromatin-defective mutants restore recombination by altering chromatin structure to one more permis-

sive for meiotic DSB formation, it also should be noted that heterochromatin appears to be a poor template for DSB repair in the absence of accessory factors. During mitosis, the Swi2 and Swi5 proteins are recruited to the silent mating-type interval in a cell type-specific manner, and are required for *mat2* or *mat3* to be efficiently used as donors in mating-type switching (Jia et al. 2004). It is not known if these or other accessory factors are present at the silent mating-type interval or at centromeres during meiosis. Thus, it remains to be determined if heterochromatin represses meiotic recombination simply by blocking DSB formation, or if it also acts to prevent the use of heterochromatic regions as templates for DSB repair by interhomolog recombination.

3.4

Areas for Future Study

While the community studying meiotic recombination in *S. pombe* is small, considerable progress has been made toward defining the relationship between chromatin structure/dynamics and recombination initiation. In particular, *ade6-M26* is arguably the best characterized of any meiotic recombination hotspot in any organism, but it remains to be seen how much of what has been learned from *M26* can be generalized to other *S. pombe* DSB hotspots (discussed in Steiner and Smith 2005). The current genome-wide identification of DSB hotspots in *rad50S* mutants (Cromie et al. 2007) should facilitate future study, especially given a recent demonstration that DSB patterns seen in *rad50S* mutants correspond closely to those seen in wild type over the entire genome (Hyppa, Cromie, and Smith, personal communication). It will be particularly important to examine the mechanism by which DSBs promote recombination in *S. pombe*, since crossovers appear to be much more evenly distributed than are DSBs (Young et al. 2002).

S. pombe is especially attractive as an organism for study, in that it displays features of chromatin and chromosome structure that are common in higher eukaryotes but are not observed in budding yeast (see Sect. 4). The presence of large regions of “classic” heterochromatin in the *S. pombe* genome, in combination with the ability to abrogate aspects of heterochromatin function by mutation, provides an excellent opportunity to understand the impact of heterochromatin on both DSB formation and the subsequent steps of meiotic recombination. Moreover, DSB patterns seen in *S. pombe*, with DSB hotspots being separated by DSB-cold regions that contain many genes, are reminiscent of the distribution of recombination hotspots seen in mammals and plants, as is the relatively large distance between DSB hotspots and protein-coding genes (see Sect. 4 and May, Slingsby and Jeffreys, this series). Thus, understanding mechanisms responsible for DSB distributions in *S. pombe* may yield important insight into the control of meiotic recombination initiation in higher eukaryotes.

4

Hints from Multicellular Organisms

Over 30 years ago, Thuriaux observed that the range of total genetic map distances amongst eukaryotes (about tenfold) was considerably less than the range in haploid genome size (about 10000-fold), and suggested that this could be explained if meiotic recombination is confined to single-copy, protein-encoding sequences, which he referred to as “structural genes” (Thuriaux 1977). This remarkably prescient suggestion provides a possible answer to the following question. If recombination directs homolog recognition and association during meiosis, how do organisms with complex genomes avoid the chromosome mispairing and genome rearrangement that would accompany recombination between dispersed repeats? One way to accomplish this would be to ensure that meiotic recombination initiates only in single-copy sequences.

While it is attractive to suggest that single-copy sequences contain unique chromatin structures that form sites for recombination initiation, there is currently insufficient data on any organism with a complex genome to support this suggestion. While recombination mapping is the subject of intense current effort, most studies have focused on mapping crossovers, which are many steps removed from the initial events of meiotic recombination. Efforts to localize earlier steps in meiotic recombination have involved, for the most part, cytological studies that are of limited resolution (see Ashley, and May, Slingsby and Jeffreys, this series). For the most part, the location of initiating events in organisms with complex genomes must be inferred from recombination maps, which in most organisms lack the resolution to relate recombination events to molecular aspects of chromatin and chromosome structure. However, high-resolution recombination maps are available in some organisms, and lower-resolution data also exist regarding the distribution of meiotic recombination in relation to large-scale features of chromosome structure. The sections below summarize current knowledge on possible relationships between chromatin structure and meiotic recombination in multicellular organisms.

4.1

Recombination Deserts

The most striking example of a region where recombination does not appear to occur is pericentromeric heterochromatin. Mapping studies in a variety of organisms have shown that, as in *S. pombe*, markers that flank these large regions of repeated sequences rarely display crossing-over (see May, Slingsby and Jeffreys, this series; Beadle 1932; Drouaud et al. 2006; Mézard 2006). Genetic studies in maize, whose genome is mostly composed of heterochromatinized transposon remnants, indicate that meiotic recombination is also

rare in interstitial heterochromatin, and is concentrated in single-copy coding sequences (Civardi et al. 1994; Fu et al. 2002; Yao et al. 2002). While this might reflect the exclusion of DSBs from heterochromatin, it is also possible that DSBs forming in these regions are rarely repaired as interhomolog crossovers. Studies of early intermediates in DSB formation and repair, detected in electron micrographs as structures called early nodules and by light microscopy as Rad51 foci, are somewhat ambiguous as to whether or not DSBs form in heterochromatin. In some organisms, early nodules appear to be excluded from pericentric heterochromatin, while studies of other organisms report detecting early nodules and Rad51 foci in these regions (Anderson et al. 2001; Anderson and Stack 2005; Barlow et al. 1997; Baudat et al. 2000; Sherman and Stack 1995; Stack 1984). This ambiguity may be due to differences between species, but may also reflect uncertainty inherent in the cytological detection and localization of both early nodules and of heterochromatin.

In summary, the best available data indicate that heterochromatin is generally nonpermissive for meiotic recombination. Whether this is due to distinct structural features of heterochromatin, or to the specific sequences that underlie heterochromatin remains to be determined; of particular interest in this regard will be the examination of *Arabidopsis* mutants that have defects in pericentric heterochromatin assembly and maintenance (Topp and Dawe 2006).

4.2

Recombination Suppression by DNA Methylation in Filamentous Fungi

In addition to heterochromatin formation, methylation of cytosine bases in DNA presents a second possible mechanism for suppressing meiotic recombination in repeated sequences. In a number of organisms, including the filamentous fungi *Neurospora crassa* and *Ascobolus immersus* (but not in the yeasts discussed above), repeated sequences are recognized by poorly understood mechanisms, and cytosines within these repeats (and, in some cases, in flanking sequences) are methylated, often leading to gene silencing and heterochromatin formation (reviewed by Bender 1998). In *Neurospora*, a vigorous methylation-directed cytosine to thymine mutation system causes rapid divergence, rendering formerly homologous repeats unsuitable substrates for recombination (reviewed by Galagan and Selker 2004). In *Ascobolus*, where repeated sequences are methylated but not mutated, cytosine methylation is associated with meiotic recombination suppression in at least two ways. In crosses where the *b2* locus is methylated in one parent but not the other, unidirectional transfer of methylation to the unmethylated locus is observed in meiotic progeny; these transfers display properties (including frequency and distribution in *b2*) expected for gene conversion events (Colot et al. 1996). One interpretation of the unidirectional nature of these events (the unmethylated locus is converted to methylated, but not vice versa) would suggest

that DNA methylation prevents DSB formation, either directly or by promoting heterochromatin assembly. However, DNA methylation clearly affects other steps in meiotic recombination after DSB formation, as the frequency of crossing over in *b2* in a methylated/unmethylated cross is reduced about 50-fold, while methylation transfer occurs at frequencies about two- to four-fold less than is seen for gene conversion in crosses where both parents are unmethylated (Maloisel and Rossignol 1998). Thus, while it is likely that DNA methylation substantially reduces DSB formation, it remains possible that DSBs still form in methylated DNA, and that methylation interferes with later steps in recombination.

4.3

Recombination Hotspots

The recent assembly of whole-genome sequences and of high-density sequence polymorphism maps has allowed recombination events to be mapped in humans and mice at unprecedented resolution, using both linkage disequilibrium and single-progeny analyses (discussed extensively by May, Slingsby and Jeffreys, this series; Coop et al. 2008; Kauppi et al. 2004, 2007; Myers et al. 2005; Shifman et al. 2006). Recent progress in fine-structure crossover mapping has also been made in *Arabidopsis* (Drouaud et al. 2006; Singer et al. 2006). The recombination landscapes emerging from all three species contain intense, highly localized crossover hotspots separated by 50–100-mb regions of low recombination activity. This pattern is remarkably similar to the relatively sparse distribution of DSB sites in *S. pombe* and is unlike the denser DSB distribution seen in *S. cerevisiae*. Hotspot maps, determined primarily in humans from linkage disequilibrium data, show that most crossover hotspots are tens of kilobases from the transcription start site of the nearest protein-coding gene (Myers et al. 2005). Recent pedigree studies indicate that a substantial fraction of human meiotic crossovers do not occur at hotspots identified by linkage disequilibrium, and also confirm the conclusion that most crossovers occur far from the nearest transcription start site (Coop et al. 2008).

Thus, while most crossover hotspots appear to be located in single-copy sequences, their distribution relative to transcription promoters more closely resembles the picture seen in *S. pombe* than that seen in *S. cerevisiae*, where most DSB hotspots are immediately 5' to protein-coding sequences. If chromatin structure plays a role in determining where meiotic recombination initiates in humans and other multicellular eukaryotes, the most "open" sites may be present far from transcription promoters (as exemplified by the locus control regions; reviewed by Dean 2006), or there may be additional factors that direct recombination initiation to particular sites. In this regard, association studies have identified a sequence motif that is associated with a minor fraction of recombination hotspots, and a sequence change within this mo-

tif at the *DNA2* and *NID1* recombination hotspots is linked to loss of hotspot activity (Myers et al. 2005; discussed in May, Slingsby and Jeffreys, this series). However, there are also cases where hotspot activity is polymorphic at a locus without a change in underlying DNA sequence, indicating that recombination activity sometimes can be controlled epigenetically by transmissible features of chromatin structure, or by distant or unlinked modifier loci (Baudat and de Massy 2007; Neumann and Jeffreys 2006; Shiroishi et al. 1991).

4.4

Areas for Future Research

In summary, current data indicate that the distribution of recombination hotspots in higher organisms, most particularly in humans, is inconsistent with the *S. cerevisiae*-derived picture of an opportunistic DSB-forming machinery that makes general use of open sites in chromatin that are located in transcription promoters of protein-encoding genes. Instead, meiotic recombination in higher organisms appears to occur at a limited number of sites whose features, at least those specifically relevant to recombination initiation, are obscure. It remains to be determined if these sites are hotspots because they contain specific sequence elements, special chromatin components or structures, or by virtue of a favorable spatial relationship with critical chromosome structures. Distinguishing between these and other possibilities is unlikely to be accomplished without direct molecular analyses.

The relatively low density of recombination events in higher eukaryotes, even at the strongest recombination hotspots, and the limited amount of meiotic material available for direct analysis in most multicellular organisms combine to make such analysis a real challenge. However, recent methodological developments, in particular the application of high-throughput sequencing strategies to whole-genome analysis of chromatin structure (Rando 2007), hold out the hope that many molecular approaches that have been used in the study of meiosis in budding and fission yeast can be applied, with suitable modification, to the study of the molecular events of recombination in higher eukaryotes. Such efforts, if they are to be successful, must be performed in the context of experimental systems and organisms where global patterns of meiotic recombination are understood at high resolution, and where the relationship between individual loci and features of meiotic chromosome structure can be determined. Thus, future progress in the understanding of the control of meiotic recombination, and its relationship to chromatin and higher-order chromosome structure, will require not only new techniques to probe chromatin and chromosome structure, but also progress in what have historically been the two mainstays of the field: high-quality genetic maps and high-resolution chromosome cytology.

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Meiotic Recombination in *Schizosaccharomyces pombe*: A Paradigm for Genetic and Molecular Analysis

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Abstract The fission yeast *Schizosaccharomyces pombe* is well-suited for both genetic and biochemical analysis of meiotic recombination. Recent studies have revealed ~50 gene products and two DNA intermediates central to recombination, which we place into a pathway from parental to recombinant DNA. We divide recombination into three stages – chromosome alignment accompanying nuclear “horsetail” movement, formation of DNA breaks, and repair of those breaks – and we discuss the roles of the identified gene products and DNA intermediates in these stages. Although some aspects of recombination are similar to those in the distantly related budding yeast *Saccharomyces cerevisiae*, other aspects are distinctly different. In particular, many proteins required for recombination in one species have no clear ortholog in the other, and the roles of identified orthologs in regulating recombination often differ. Furthermore, in *S. pombe* the dominant joint DNA molecule intermediates contain single Holliday junctions, and intersister joint molecules are more frequent than interhomolog types, whereas in *S. cerevisiae* interhomolog double Holliday junctions predominate. We speculate that meiotic recombination in other organisms shares features of each of these yeasts.

Abbreviations

DSB	double-strand break
HJ	Holliday junction
LinE	linear element
MCM	mini-chromosome maintenance
MI	first meiotic division
MMR	mismatch repair
MRN	Mre11-Rad50-Nbs1 complex
NER	nucleotide excision repair
SC	synaptonemal complex
SDSA	synthesis-dependent strand annealing
SPB	spindle-pole body
ss	single-stranded
SSB	ss DNA binding protein

1

***S. pombe*: An Excellent Model Organism for Studying Meiotic Recombination**

Homologous genetic recombination plays two important roles during meiosis, the special nuclear divisions during which chromosome number is reduced from two (diploid) to one (haploid). First, recombination provides the physical connection between homologs that aids their pairing and proper segregation at the first meiotic division (MI), and second, it increases the genetic diversity that aids evolution (see Lankenau, this book). Elucidating the molecular mechanism of meiotic recombination requires a combination of genetic and biochemical analysis. Fungi, such as yeasts, have been particularly useful in this regard, for they have the essential features of meiosis found in complex organisms yet are more tractable for genetics and biochemistry. Notably, in many fungi the haploid products (spores) from each meiosis are enclosed in an ascus. Analysis of the haploid progeny from one ascus reveals all of the products of a single meiotic recombination event at each locus analyzed.

Meiosis has been especially well-studied in the budding yeast *Saccharomyces cerevisiae* (see Keeney, this SERIES; see chapters by Heyer; Lichten; Hunter, this book) and the distantly related fission yeast *Schizosaccharomyces pombe* discussed here (see also Ding & Hiraoka, this book; see further Pérez-Hidalgo, Moreno and Martín-Castellanos; or Tanaka and Watanabe, this SERIES). Both haploid and diploid cells of these yeasts can be grown indefinitely by mitotic division; genetic analysis that uses recessive markers is simpler in haploids. Large cultures of cells can be synchronously induced for meiosis, facilitating biochemical analysis. The nucleotide sequences of their relatively small genomes, ~14 Mb, are essentially complete, permitting comprehensive genomic studies. In addition, *S. pombe* offers special advantages. The strongest meiotic recombination-deficient (Rec^-) mutants of *S. pombe* produce many viable spores in part because this species has only three chromosomes, which, in the absence of recombination, would still be expected to segregate correctly and produce viable spores 12.5% of the time (2^{-3}). *S. pombe* also has a mechanism for actively segregating non-recombinant (achiasmate) chromosomes at MI (Molnar et al. 2001; Davis and Smith 2005). Consequently, strong Rec^- mutants that cannot initiate recombination are nevertheless able to produce ~10–25% as many viable spores as the wild type, an outcome that greatly aids analysis of such mutants (Ponticelli and Smith 1989; Young et al. 2004; Gregan et al. 2005). All commonly used strains are derived from a single culture (Munz et al. 1989): their near isogenicity simplifies the use of strains and comparisons of results among labs. The M26 and closely related hotspots of recombination are exceptionally strong and are the best-defined meiotic hotspots in terms of nucleotide sequence (see Sect. 6.1).

Some aspects of the molecular biology of *S. pombe* are more similar to those of humans than are those of *S. cerevisiae*. These aspects of *S. pombe* include more complex centromeres and origins of replication, the presence of RNAi and certain histone modifications, and the specifics of cell-cycle control. In contrast, *S. pombe* is unusual in not having a fully developed synaptonemal complex (SC; Olson et al. 1978; Bähler et al. 1993), a large meiosis-specific structure joining paired homologs (see chapters by Suja and Julio S. Rufus, or Mehrotra, Hawley and McKim, this SERIES). The role of the SC is not clear, but its absence from *S. pombe* indicates that it is not essential for meiosis or recombination. In addition, *S. pombe* does not have crossover interference, the regulation of the number of crossovers and their distribution along chromosomes (Munz 1994). These characteristics allow in *S. pombe* a study of the essential features of recombination without the complexities of the SC or interference. Comparison of results among different species, such as *S. pombe* and *S. cerevisiae*, has revealed both conserved and diverged aspects of meiosis. In this regard, comparison of *S. pombe* and *S. cerevisiae* may help deduce the evolution of meiosis.

2

Overview: A Pathway for *S. pombe* Meiotic Recombination

In our current understanding of *S. pombe* meiotic recombination there are three stages, the first of which is concurrent with the other two: 1) the overall alignment and then intimate pairing of homologs, 2) the programmed formation of DNA double-strand breaks (DSBs), and 3) the repair of DSBs (Fig. 1). Stages 1 and 2 are meiosis-specific, whereas stage 3 shares many functions with mitotic DNA repair. Stage 1, homolog alignment, involves the clustering of telomeres (“bouquet” formation) and the movement of the nucleus back and forth in the cell (“horsetail” formation) (see Ding & Hiraoka, this book). These features are found in most organisms but are exaggerated in *S. pombe*. Homolog alignment reduces recombination between non-allelic loci with similar sequences (Niwa et al. 2000; Davis and Smith 2006); ectopic recombination between such loci could generate deleterious translocations.

During the horsetail stage chromosomes are replicated and sister chromatid cohesion is modified to allow the unique segregation of homologs at MI (see Tanaka & Watanabe, this SERIES). The meiosis-specific cohesin subunits Rec8 and Rec11 are important for the formation of linear elements, which are reminiscent of the axial elements of the SC (Lorenz et al. 2004). Linear elements, in turn, appear to be important for the assembly onto the chromosomes (or activation) of the proteins that make DSBs, including the active-site protein Rec12 (Spo11 homolog).

DSBs are made by Rec12 in concert with other proteins (stage 2) and are repaired by interaction with homologous DNA of either the sister chromatid

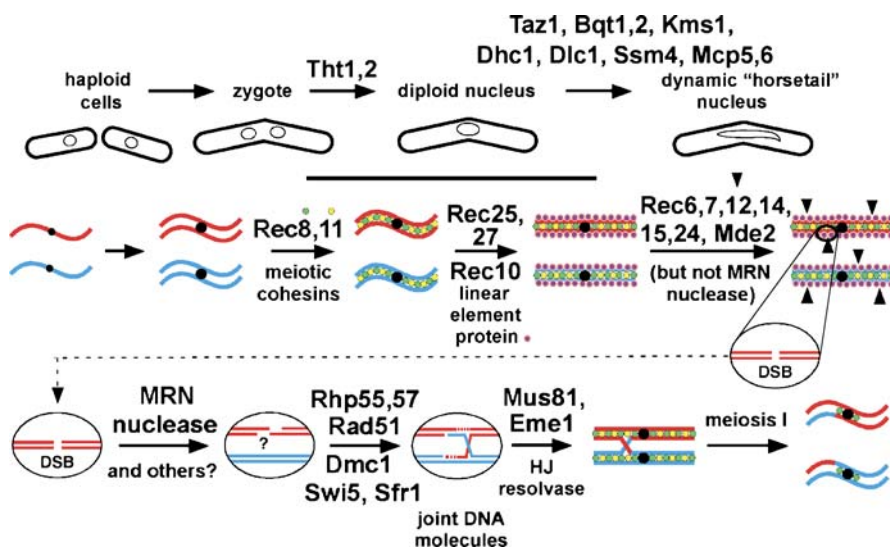


Fig. 1 A pathway for meiotic recombination in *S. pombe*. The upper panels portray the fusion of cells and nuclei and the formation of the “horsetail” nucleus. The middle and lower panels portray the chromosome and DNA events that occur during the horsetail stage, which ceases shortly before meiosis I. Identified gene products required at each stage are indicated above the arrow leading to that stage. Additional proteins required for meiotic recombination, but whose points of action are not clear, include *rec13*, *rec17* – *21*, *mug1*, *mug5*, *pds5*, *rqh1*, and *meu13* (see sections 2, 3, and 8). MRN, Rad32 (Mre11)-Rad50-Nbs1 complex. Modified from Ellermeier and Smith (2005)

or the homolog (stage 3). Only interhomolog interaction gives rise to the physical connections (chiasmata) that aid homolog segregation at MI, but sister chromatid exchange does occur. The regulation of these two types of repair is an intriguing problem not yet solved. DSB repair occurs in steps. First is the formation of hybrid DNA, which has one strand from each parental DNA, and one or two Holliday junctions, an intermediate with two crossed, single strands connecting the parental duplexes. Second is the resolution of the Holliday junction(s) into linear duplexes, which may occur in either the crossover or non-crossover configuration. Regulation of this outcome is also an intriguing, largely unsolved problem.

In the following sections we discuss each of these stages of meiotic recombination. Tables 1–3 list the known *S. pombe* gene products required for wild-type levels of meiotic recombination. (Most of the primary references are given in these tables rather than in the text.) These gene products are listed according to the stage at which they play the most prominent role; however, some may act at more than one stage. Mutations in additional genes (*rec13* and *rec17* – *rec21*) reduce meiotic recombination frequencies (DeVeaux

et al. 1992), but these mutations have not been placed in genes identified otherwise.

S. pombe typically grows as haploid cells. When cells of opposite mating type meet under starvation conditions, the cells and then their nuclei fuse to form a diploid. Unless nutrients are supplied, the diploid immediately undergoes meiosis (see Pérez-Hidalgo, Moreno & Martín-Castellanos, this SERIES). Two mutants, *tht1* and *tht2*, are deficient in nuclear fusion and produce essentially no interhomolog recombinants among spores. Although not reported, intersister recombination in these mutants is expected to be high, since DSBs are formed and repaired as in wild-type. Each nucleus undergoes an aberrant meiosis, sometimes with only one nuclear division.

3

Nuclear Movement Promotes Chromosome Alignment: “Bouquet” and “Horsetail” Formation

Before the outset of meiosis the centromeres are clustered at the spindle-pole body (SPB), the fungal equivalent of the centrosome. As meiosis proceeds, the telomeres cluster and replace the centromeres at the SPB, to form the meiotic “bouquet” arrangement of chromosomes (Chikashige et al. 1994). The SPB leads the nucleus back and forth in movement across the cell, and the nucleus becomes elongated and curved, like a horse’s tail. Meiotic recombination is reduced by a factor of ~ 5 in all of the tested mutants deficient in bouquet or horsetail formation (Table 1). In these mutants DSBs are formed and, where tested, repaired with nearly wild-type frequency and kinetics. Presumably, repair occurs most frequently by interaction with the sister chromatid, with some residual interhomolog interaction accounting for the observed recombinants.

Bouquet formation requires two meiosis-specific gene products, Bqt1 and Bqt2. These small proteins appear to act as a complex, gluing the telomeres to the SPB. Throughout the life cycle, Taz1 binds to telomeres and to Rap1. The Bqt1-Bqt2 complex forms a meiosis-specific bridge between Rap1 and Sad1, a component of the SPB, thereby joining the telomeres to the SPB. In the absence of Taz1, Rap1, Bqt1, or Bqt2 the nucleus moves but, since the telomeres are not attached to the SPB, the nucleus does not assume the characteristic horsetail shape, and chromosomes are not properly aligned.

The bouquet restricts ectopic recombination, which can cause deleterious genome rearrangements. In *S. pombe*, ectopic recombination occurs 10–1000 times less frequently than allelic recombination (Virgin and Bailey 1998). Mutations in *kms1* and *bqt2* affect bouquet formation and increase the frequency of meiotic ectopic recombination up to 20-fold. Attachment of telomeres to the SPB during bouquet formation may restrict recombination to sequences

Table 1 Genes required for recombination and nuclear fusion, nuclear movement, bouquet formation, or chromosome alignment

Gene	Protein size (kDa)	Approx. extent of reduction by mutation	Putative <i>S. cerevisiae</i> ortholog (~% identity)	Inferred primary activity	Refs. for role in recombination
<i>tht1</i>	63	– ^a	Kar5 (19)	Nuclear fusion	Tange et al. 1998
<i>tht2</i>	23	1000 ^a	– ^b	Nuclear fusion	Martín-Castellanos et al. 2005
<i>taz1</i>	75	5	Tbfl (30)	Binds telomere repeats	Cooper et al. 1998; Niwa et al. 2000
<i>rap1</i>	80	– ^c	Rap1 (22)	Binds Taz1	Chikashige and Hiraoka 2001; Kanoh and Ishikawa 2001
<i>bqt1</i>	12	5	–	Connects telomeres and SPB	Martín-Castellanos et al. 2005; Chikashige et al. 2006
<i>bqt2</i>	14	5	–	Connects telomeres and SPB	Martín-Castellanos et al. 2005; Chikashige et al. 2006; Davis and Smith 2006
<i>sad1</i>	58	– ^c	Mps3 (17)	SPB component	Hagan and Yanagida 1995
<i>kms1</i>	69	5	–	SPB component	Shimanuki et al. 1997; Miki et al. 2002
<i>mcp6</i>	38	5	–	SPB component	Saito et al. 2005
<i>dhc1</i>	484	5	Dyn1 (25)	Dynein motor protein	Yamamoto et al. 1999
<i>dlc1</i>	12	10	YER071C (21)	Dynein accessory factor	Miki et al. 2002
<i>ssm4</i>	77	10	Nip100 (24)	Binds Dhc1	Niccoli et al. 2004
<i>mcp5 (num1)</i>	111	5	Num1 (24)	Binds dynein to microtubules	Saito et al. 2006; Yamashita and Yamamoto 2006; C. Ellermeier, pers. comm.

Table 1 (continued)

Gene	Protein size (kDa)	Approx. extent of reduction by mutation	Putative <i>S. cerevisiae</i> ortholog (~% identity)	Inferred primary activity	Refs.
<i>pds5</i> ^d	139	5	Pds5 (24)	Sister chromatid cohesin partner	Tanaka et al. 2001; Wang et al. 2002; Ding et al. 2006; unpublished data
<i>rql1 (rec9)</i> ^d	150	5	Sgs1 (37)	RecQ family helicase	Stewart et al. 1997; J. Young, pers. comm.
<i>mug1</i> ^d	41	5	Uso1 (23) Sla2 (23)		Martín-Castellanos et al. 2005; C. Ellermeier, pers. comm.
<i>mug5</i> ^d	21	5	-		Martín-Castellanos et al. 2005; C. Ellermeier, pers. comm.

^a *tht1* is recombination-proficient in azygotic meiosis (that of an established diploid); *tht2* was not tested. *tht1* is assumed to be as deficient in zygotc meiosis (that immediately following mating) as *tht2*

^b No ortholog is obvious

^c Not determined. Requirement for meiotic recombination is assumed, based on the protein being required for telomere clustering during meiosis

^d Role in recombination is uncertain. See section 3

equivalent distances from the anchored telomeres. This spatial constraint would favor allelic over ectopic recombination.

Horsetail movement requires the dynein components Dhc1 (heavy chain) and Dlc1 (light chain), the dynactin component Ssm4, and the SPB components Mcp6 (meiotic coiled-coil protein) and Kms1. During meiosis the SPB is linked to the dynein motor complex via Kms1 and perhaps the dynactin complex. Dynein is the motor that moves the nucleus, led by the SPB, along the microtubule arrays in the cell. In *dhc1*, *dlc1*, and *ssm4* mutants, the nucleus does not move and the homologs do not align, although the telomeres become attached to the SPB. Attachment of dynein to microtubules at the cell cortex, which generates the force for horsetail movement, requires Mcp5 (Num1).

Additional genes are placed at the bottom of Table 1 because the corresponding mutants produce recombinant frequencies ~5 times lower than that of wild type and, where tested, make and repair DSBs with nearly wild-type kinetics and frequencies, as is the case for the bouquet- and horsetail-defective mutants previously discussed. Pds5 aids loading of the Rec8 cohesin subunit (see sections 4 and 5.2); in *pds5* mutants the chromosomes are hyper-compacted, and horsetail shape is aberrant. Rqh1 is a homolog of the *E. coli* RecQ and *S. cerevisiae* Sgs1 helicases; *rqh1* was identified (as *rec9*) in the initial screen for *S. pombe* meiotic Rec⁻ mutants (Ponticelli and Smith 1989). *mug1* and *mug5* are meiotic up-regulated genes; the mutants make aberrant asci indicative of chromosome missegregation. Further analyses are required to determine the stage at which these proteins promote recombination.

4

Meiosis-specific Sister Chromatid Cohesins: Behavior Change

During or shortly after meiotic replication, the meiosis-specific cohesin subunits Rec8 and Rec11 are recruited to the chromosomes, where they largely replace the mitotic cohesin subunits Rad21 and Psc3 (see Tanaka & Watanabe, this SERIES). During mitotic division Rad21 is cleaved by separase (Cut1) to allow sister chromatid segregation. During the first meiotic division, Rec8 located in the chromosome arms is, like Rad21, cleaved by separase. However, unlike Rad21, Rec8 at the centromeres is protected from separase by Sgo1 (Kitajima et al. 2004). This differential cleavage allows sisters to separate distal to the crossovers that hold homologs together but maintains cohesion between sisters at the centromeres (Fig. 1). Thus, the change in cohesins permits segregation of homologs, rather than sisters, at MI. The role of Rec11 is less clear; its location primarily in the arms suggests involvement in arm cohesion, but, as noted in Sect. 5.3, Rec11 and Rec8 are also required for recombination.

5

DSB Formation by Rec12: Preparation and Partnership

5.1

***S. pombe*: A Second Eukaryote with Directly Observed Meiotic DSBs**

DSBs were postulated to initiate meiotic recombination (Resnick 1976; Szostak et al. 1983) many years before their demonstration in *S. cerevisiae* at hotspots of recombination (Sun et al. 1989; Cao et al. 1990). Searches for DSBs in *S. pombe* were first successful when whole chromosomes and large restriction fragments were examined (Cervantes et al. 2000). Aided by a mutant, *rad50S* (see Sect. 7.1), DSBs were later found at the genetically well-characterized hotspot *M26* (Steiner et al. 2002; see Sect. 6.1). Meiotic DSBs have not, to our knowledge, been directly observed in other organisms. They have been inferred, however, from the requirement for Spo11 homologs for successful meiosis or recombination, from the Spo11-dependent fragmentation of chromosomes in DSB repair-deficient mutants (Pasierbek et al. 2001; Puizina et al. 2004), or from the appearance on meiotic chromosomes of foci of a particular form of histone H2 that is thought to be a signal of DSBs (see Lichten, this book). The direct detection of DSBs in *S. pombe* opened the way for the discovery of natural *S. pombe* hotspots, discussed below, and the study of other intermediates of recombination.

5.2

Modification of Sister Chromatid Cohesion:

A Foundation for Meiosis-specific DSB Formation

As noted above, the substitution of the Rec8 and Rec11 cohesin subunits for their Rad21 and Psc3 mitotic counterparts dramatically modifies the segregation behavior of chromosomes during meiosis. Rec8 and Rec11 also initiate a series of events that lead to meiotic DSBs. Current evidence indicates that, after Rec8 and Rec11 are placed on chromosomes, the Rec25 and Rec27 proteins, perhaps as a complex, form foci on the chromosomes. In turn, these proteins allow the loading of Rec10, a major component of linear elements (Lorenz et al. 2004; see Sect. 5.3). Finally, Rec7 and presumably the other proteins required for DSB formation, including Rec12, are recruited to the chromosomes (Lorenz et al. 2006). Thus, the modification of chromosomes both for their unique segregation and for high-level recombination appears to be initiated at the time of meiotic replication (see Sect. 4).

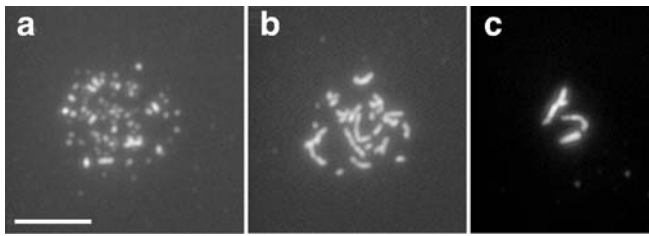


Fig. 2 Linear elements containing Rec10 protein. Spreads of meiotic nuclei were stained using an antibody to Rec10 protein. Dots (**a**), filaments (**b**), and bundles (**c**) appear to occur in that order during meiosis. The filaments and bundles appear to reflect the linear elements seen by electron microscopy (see text). Bar indicates 5 μm . Figure supplied by J. Loidl

5.3

Formation of Linear Elements:

Structures Reminiscent of the Synaptonemal Complex

Electron microscopy of thin sections of *S. pombe* meiotic cells or of spreads of their nuclear contents fail to reveal the synaptonemal complexes (SC) common to most organisms (Olson et al. 1978; Bähler et al. 1993; Loidl 2006). Structures similar to one part of the SC, however, are observed and are designated linear elements (LinEs; Bähler et al. 1993; Lorenz et al. 2004). The classical SC is composed of a central element between two parallel lateral elements connected by transverse filaments when homologs are fully aligned and intimately paired. Before this pairing, the lateral elements are called chromatid cores or axial elements; they encase the bases of the chromatin loops of each sister chromatid pair (see chapters by Suja and Julio S. Rufus, or Mehrotra, Hawley and McKim, this SERIES). The LinEs of *S. pombe* appear similar to axial elements, but LinEs do not show the parallel alignment of lateral elements in paired chromosomes and do not extend the full length of the chromosomes as do the axial elements of the SC (Bähler et al. 1993). The role of LinEs, like that of the SC, is not clear, but *rec8* and *rec10* mutants have aberrant or no LinEs, respectively, and are recombination-deficient (Molnar et al. 1995, 2003; Lorenz et al. 2004; Loidl 2006).

Fluorescence microscopy of meiotic cells or nuclear spreads reveals structures likely identical to the LinEs, and this analysis confirms the close connection between LinEs and recombination. During meiosis the LinE component Rec10 first forms discrete nuclear foci and then filaments (Fig. 2), whose numbers and morphological classes approximate those of the LinEs seen by electron microscopy. Formation of Rec10 filaments requires Rec8, Rec11, Rec25, and Rec27 (Lorenz et al. 2004; C. Martín-Castellanos, personal communication). All of these proteins are required for full levels of recombina-

nation, presumably by allowing the loading or activation of the DSB-forming complex.

5.4

Rec12: The Active Site Protein for DSB Formation

Meiotic DSBs are formed by Rec12, the *S. pombe* homolog of Spo11, in conjunction with other proteins. *S. cerevisiae* Spo11 becomes covalently linked to the 5' ends of the DNA at a DSB, presumably via a tyrosine residue that is essential for DSB formation and recombination (Keeney 2001). The corresponding tyrosine in Rec12 is also required for DSB formation and recombination (Cervantes et al. 2000), and covalent linkage of Rec12 to DNA has been inferred from chromatin-immunoprecipitation studies using an epitope-tagged version of Rec12 (R. Hyppa, pers. comm.). Spo11 homologs from a wide variety of organisms, including *S. pombe* Rec12, have amino acid sequences similar to that of an archeal DNA topoisomerase, whose crystal structure reveals a dimer with the two active-site tyrosine residues pointed into a cleft plausibly holding DNA during catalysis of DSB formation (Nichols et al. 1999). Thus, the mechanism of meiotic DSB formation appears to be highly conserved and closely related to that of type II topoisomerases (see Keeney, this SERIES).

5.5

Other Proteins Essential for DSB Formation: Potential Rec12 Partners and Regulators

Rec12 does not make DSBs on its own but requires numerous other proteins. The cascade of proteins noted in Sections 5.2 and 5.3 appear to be needed for the proper localization of Rec12, and other proteins are needed for Rec12 activity. *rec12* mutants have no detectable meiotic recombination above the level in mitotic cells and no detectable DSBs (Young et al. 2002). This is also true for *rec6* and *mde2* mutants (Table 2), indicating that their gene products are essential for Rec12 action. The corresponding proteins may be partners for Rec12, perhaps in a complex with it, much as several proteins activate *S. cerevisiae* Spo11 by forming a complex with it (see Keeney, this SERIES). Loading of Rec7 requires Rec10 (Lorenz et al. 2006) and presumably also the proteins needed for Rec10 loading (Rec8, Rec11, Rec25, and Rec27; see above). It is noteworthy that the MRN complex (see Sect. 7.1) is not required for DSB formation in *S. pombe*, although its homolog MRX in *S. cerevisiae* is required (Cao et al. 1990; Young et al. 2004); in both organisms the complex is required for DSB repair. Other differences in the control of DSB formation and repair are discussed in Sect. 13.

In *S. cerevisiae* meiotic replication is essential for DSB formation (Borde et al. 2000; Smith et al. 2001), but in *S. pombe* the situation is less clear. As in *S. cerevisiae*, DSBs appear after replication (Cervantes et al. 2000), but repli-

Table 2 Genes required for recombination and meiosis-specific sister chromatid cohesion, linear element formation, or DSB formation

Gene	Protein size (kDa)	Approx. extent of reduction by mutation	Putative <i>S. cerevisiae</i> ortholog (~% identity)	Inferred primary activity	Refs. for role in recombination
<i>rec8</i>	64	5–500 ^a	Rec8 (20)	Sister chromatid cohesion	Ponticelli and Smith 1989; Parisi et al. 1999; Ellermeier and Smith 2005
<i>rec11</i>	107	5–500 ^a	Irr1(Scc3) (20)	Sister chromatid cohesion	Ponticelli and Smith 1989; Ellermeier and Smith 2005
<i>rec25</i>	17	15	– ^b	Aids linear element formation	Martin-Castellanos et al. 2005
<i>rec27</i>	16	15	–	Aids linear element formation	Martin-Castellanos et al. 2005
<i>rec10</i>	90	1000	Red1 (v. limited) ^c	Linear element component	Ponticelli and Smith 1989; Lorenz et al. 2004; Ellermeier and Smith 2005
<i>rec12</i>	39	1000	Spo11 (30)	Makes DSBs	DeVaux et al. 1992; Cervantes et al. 2000
<i>rec6</i>	21	1000	–	Putative Rec12 partner	Ponticelli and Smith 1989; Cervantes et al. 2000
<i>rec7</i>	38	1000	Rec114 (9)	Putative Rec12 partner	Ponticelli and Smith 1989; Cervantes et al. 2000; Molnar et al. 2001
<i>rec14</i>	33	1000	Ski8 (25)	Putative Rec12 partner	DeVaux et al. 1992; Cervantes et al. 2000

Table 2 (continued)

Gene	Protein size (kDa)	Approx. extent of reduction by mutation	Putative <i>S. cerevisiae</i> ortholog (~% identity)	Inferred primary activity	Refs. for role in recombination
<i>rec15</i>	21	1000	-	Putative Rec12 partner	DeVeaux et al. 1992; Cervantes et al. 2000
<i>rec24</i>	40	1000	-	Putative Rec12 partner	Martín-Castellanos et al. 2005
<i>mde2</i>	23	300	-	Putative Rec12 partner	Gregan et al. 2005
<i>hsk1</i>	58	10	Cdc7 (37)	Protein kinase; substrate unknown	Ogino et al. 2006
<i>atf1</i>	60	15 ^d	Sko1 (25)	Stress response transcription factor	Kon et al. 1997; Fox et al. 2000
<i>pcr1</i>	19	15 ^d	-	Stress response transcription factor	Kon et al. 1997; Fox et al. 2000

^a The extent of reduction depends on the interval measured. See section 6.3.

^b No ortholog is obvious.

^c A region of 64 amino acids has ~27% identity.

^d Reduction only at *M26* and related hotspots.

cation can be severely inhibited by mutations or by hydroxyurea with only slight diminution of DSB formation, provided the replication checkpoint is inactivated (Tonami et al. 2005). Hydroxyurea blocks transcription of several meiotic genes required for DSB formation, including *mde2*, and replication checkpoint mutations relieve this block (Ogino and Masai 2006). Conversely, a particular *hsk1* mutant does not form detectable DSBs under conditions in which meiotic replication appears normal (Ogino et al. 2006). Hsk1 protein kinase is required for mitotic replication, via phosphorylation of the MCM (mini-chromosome maintenance) complex; the *hsk1* mutant tested may lack a second function needed for DSB formation, such as phosphorylation of Rec12 or one or more of its putative partners. Meiotic replication and DSB formation in *S. pombe* may be normally coupled by a checkpoint mechanism but not obligatorily coupled as appears to be the case in *S. cerevisiae*.

6

DSB Hotspots and Coldspots: Regulating Where Recombination Occurs

Rec12 does not make DSBs uniformly across chromosomes; rather, there are sites or regions with DSBs at above-average frequency (hotspots) and below-average frequency (coldspots). Hotspots and coldspots were first identified genetically as chromosomal intervals with higher or lower than average intensity of recombination (Gutz 1971; see May, Slingsby and Jeffreys, this SERIES). Wild-type chromosomes in all organisms tested have such hot and cold intervals, but the *S. pombe* mutation *ade6-M26*, which creates a hotspot, has been especially informative.

6.1

M26: A Eukaryotic Sequence-specific Hotspot

The *ade6-M26* mutation recombines with other *ade6* mutations ~10 times more frequently than does the closely linked *M375* mutation (Gutz 1971). By tetrad analysis *M26* also converts ~10 times more frequently than does *M375*, and it converts preferentially to *ade6*⁺. *M26* is a single bp mutation G → T that creates the sequence 5' ATGACGT 3', each nucleotide of which is important for hotspot activity (Ponticelli et al. 1988; Szankasi et al. 1988; Schuchert et al. 1991). This sequence is bound by the Atf1-Pcr1 "stress response" transcription factor, which is essential for *M26* hotspot activity (Wahls and Smith 1994; Kon et al. 1997). An iterative binding and PCR-amplification scheme identified 5' GNVTATGACGTCATNBNC 3' as a consensus sequence for Atf1-Pcr1 binding to DNA, and mutations creating this sequence in *ade6* have hotspot activity greater than that of *M26* itself (Steiner and Smith 2005b). Sequences closely related to this consensus occur in the wild-type *S. pombe* genome, and the majority of 15 such loci tested are hotspots of DSB for-

mation (Steiner and Smith 2005a). In the one case tested, in the *cds1* gene, this sequence is also a hotspot of recombination. This appears to be the first case of meiotic recombination hotspots being successfully predicted from a genome's sequence. [In *S. cerevisiae*, sites bound by the Bas1 transcription factor are hot- or coldspots (Mieczkowski et al. 2006).] Collectively, the *M26*-like sequences may account for a few percent of all of the meiotic DSBs and recombination. Other transcription factors may account for additional DSBs and recombination.

The molecular basis of the *M26* hotspot is partially understood. During meiosis the chromatin at the *M26* site becomes more sensitive ("open") to exogenous micrococcal nuclease in an *M26* sequence- and Atf1-Pcr1 factor-dependent manner (Mizuno et al. 1997, 2001; Yamada et al. 2004). DSB formation at and around *M26* depends on Rec12 and Pcr1 (Steiner et al. 2002). Among *M26*-like sequences, there is a strong correlation between DSB frequency and hotspot activity, leaving no doubt that these DSBs are causally related to recombination. The *M26* sequence is not sufficient, however, for hotspot activity. Most transplacements of the *ade6-M26* gene, with >1 kb of DNA to each side of *M26*, to a distant site do not manifest hotspot activity (Ponticelli and Smith 1992). Presumably, the chromatin structure is influenced by nucleotide sequences > 1 kb away from *M26* and is more "open" at the endogenous *ade6* locus. The features of "open" chromatin that permit DSB-formation are currently unknown but may involve binding of Rec12 or its putative partners to proteins that "open" the chromatin (see Lichten, this book).

6.2

Hotspots in Large Intergenic Regions: Another Role for "Junk" DNA?

Surveys for DSBs across large regions of wild-type *S. pombe* chromosomes reveal prominent DSB hotspots roughly 50–100 kb apart separated by regions with few, if any, DSBs (Young et al. 2002). Each of these hotspots appears to be a cluster of DSB sites spread over ~1–3 kb. Among 24 such prominent DSB hotspots examined, 21 fall in intergenic intervals markedly larger than the mode of 0.4 kb (Wood et al. 2002); 15 of these 21 DSB hotspots are in intergenic intervals > 4 kb, and the smallest of these 21 intervals is 1.9 kb (unpublished data). The nucleotide sequences responsible for these prominent hotspots have not been determined. They may be collections of transcription factor binding sites exemplified by *M26*, as previously discussed. Alternatively, the primary role of this apparently "junk" intergenic DNA may be to promote meiotic recombination.

6.3

Region-specific Activation by Cohesins: Megabase-scale Control of DSB Formation

Early studies showed that *rec8*, *rec10*, and *rec11* mutants are far more deficient for recombination at the *ade6* locus, the basis for their isolation (Ponticelli and Smith 1989), than in several other intervals tested (DeVeaux and Smith 1994). For example, in *rec8* Δ and *rec11* Δ mutants, *ade6* recombination is reduced by a factor of ~ 500 , whereas recombination in many other intervals is reduced by a factor of 10 or less. In *rec10* Δ mutants recombination is strongly reduced throughout the genome, although the initial mutant *rec10-109*, a double missense, behaves much like the *rec8* Δ and *rec11* Δ mutants (Ellermeier and Smith 2005). The intervals with the least reduction in *rec8* and *rec11* mutants appear to be toward the ends of the chromosomes, although no clear pattern has been established (Parisi et al. 1999). Nevertheless, the strongly affected intervals are large – up to a few Mb.

The basis for this remarkable regional specificity is not entirely clear. It is noteworthy that in a *rec8* Δ mutant, Rec10 forms short patches that may correspond to short LinEs seen by electron microscopy (Molnar et al. 1995; Lorenz et al. 2004). Rec8 and Rec11 meiosis-specific cohesin subunits are required for the cascade resulting in the loading of Rec12 (see Sect. 5). These cohesin subunits do not entirely replace the mitotic cohesin subunit Rad21, residual levels of which remain along meiotic chromosomes (Yokobayashi et al. 2003). In the absence of Rec8 or Rec11, Rec10 may be able to load onto Rad21-bound intervals and lead to DSBs in those intervals; the Rec10-109 mutant protein may be active with Rad21 but not with Rec8 or Rec11.

6.4

Recombination in DSB-poor Intervals: Action at a Distance or Novel Lesions?

Between the prominent hotspots noted in Sect. 6.2 are regions of 50–100 kb with few, if any, DSBs. Nevertheless, in the intervals tested crossovers occur at an intensity (cM per kb) close to that in intervals with prominent DSB hotspots (Young et al. 2002). The origin of these crossovers is currently unclear. The hypothesis that crossovers are generated by distant DSBs (Smith 2001; Young et al. 2002) was not supported by direct and indirect tests (Cromie et al. 2005). Perhaps there are DNA lesions other than DSBs that occur in the DSB-poor regions and lead to crossovers. If so, these lesions must depend on Rec12 and the tyrosine at its putative active site, for a mutant lacking this tyrosine is completely deficient for meiotic recombination (Cervantes et al. 2000). Rec12 may generate recombinogenic single-strand lesions, such as nicks and gaps, in some intervals and DSBs in others. Alternatively, some Rec12-dependent DSBs may not be detectable by the methods used.

6.5

Coldspots: Forbidden Regions for Recombination

Reciprocal recombination (crossing-over) occurs throughout most of the genome with nearly uniform intensity of 0.16 cM/kb (Young et al. 2002). Two regions, however, appear to have essentially no recombination. The first recognized is the 15 kb “K region” between the two silent mating-type loci, *mat2* and *mat3*, which are separated by < 0.002 cM, indicating a recombination intensity < 0.1% of the genome average (Egel 1984). Recombination also appears to be rare within centromeres: loci flanking *cenII* and *cenIII* recombine with an intensity (cM/kb) < 3% of the genome average (Nakaseko et al. 1986; C. Ellermeier, V. Tseng, and G. Smith, unpublished data).

The reduced recombination appears to be due to heterochromatin at these loci. There are multiple copies of several different repeats in the centromeres (Wood et al. 2002), and ~4.3 kb of the *mat* K region shares 96% identity with parts of two of these repeats (Grewal and Klar 1996). In the K region and at centromeres, heterochromatin blocks expression of inserted genes, and mutations that alter chromatin structure, such as *swi6* (HP-1 homolog) or *rik1* (putative partner for the Clr4 histone methyltransferase), allow both gene expression and recombination in these intervals (Egel et al. 1989; Klar and Bonaduce 1991; C. Ellermeier and G. Smith, unpublished data). Presumably, wild-type “closed” chromatin in these intervals prevents DSB formation by Rec12. The biological advantage of such cold regions is not clear, but recombination in these intervals may interfere with normal mating-type switching (K region) or chromosome segregation (centromeres) (see chapters by Kokotas, Grigoriadou and Petersen, or Tanaka & Watanabe, this SERIES).

7

Processing of Rec12-generated DSBs:

Converting a Lesion into a Recombinogenic DNA-Protein Complex

Rec12-generated DSBs must be processed into a DNA-protein complex capable of initiating strand exchange with a homologous duplex. The major steps are thought to be removal of bound Rec12 from the 5' strand termini of the DSB, resection of the 5' strands to give long 3' single-strand (ss) DNA overhangs, and loading of strand exchange proteins onto the single-stranded (ss) DNA (Table 3).

Table 3 Genes required for recombination and DSB or mismatch repair

Gene	Protein size (kDa)	Approx. extent of reduction by mutation	Putative <i>S. cerevisiae</i> ortholog (~% identity)	Inferred primary activity	Refs. for role in recombination
<i>rad32</i>	74	15	Mre11 (42)	MRN component; nuclease	Tavassoli et al. 1995
<i>rad50</i>	150	– ^a	Rad50 (34)	MRN component; ATPase	–
<i>nbs1</i>	69	–	Xrs2 (limited) ^b	MRN component	–
<i>rad51</i> ^c	40	15	Rad51 (71)	Strand exchange protein	Grishchuk and Kohli 2003
<i>dmc1</i>	36	3	Dmc1 (63)	Strand exchange protein	Grishchuk and Kohli 2003
<i>rad55</i>	39	5	Rad55 (32)	Rad51 accessory protein	Grishchuk and Kohli 2003
<i>rad57</i>	40	3	Rad57 (37)	Rad51 accessory protein	Grishchuk and Kohli 2003
<i>swi5</i>	10	5	Sae3 (24)	Rad51 and Dmc1 accessory protein	Ellermeier et al. 2004
<i>sfr1</i>	34	10	Mei5 (limited) ^d	Rad51 and Dmc1 accessory protein	Unpublished data
<i>rlp1</i>	42	2 (xo only) ^e	hXRC2 (31)	Rad51 accessory protein	Grishchuk and Kohli 2003
<i>mcp7</i>	24	10	Mnd1 (26)	Acts with Dmc1	Saito et al. 2004
<i>meu13</i>	25	10	Hop2 (27)	Acts with Dmc1	Nabeshima et al. 2001; Saito et al. 2004
<i>rad54</i>	97	1 ^f	Rad54 (54)	Chromatin remodeling	Catlett and Forsburg 2003
<i>rdh54</i>	93	3 ^f	Rdh54 (33)	Chromatin remodeling	Catlett and Forsburg 2003
<i>mus81</i>	69	50 (xo only) ^g	Mus81 (28)	Holliday junction resolvase	Osman et al. 2003; Smith et al. 2003
<i>eme1</i>	83	–	Mms4 (10)	Holliday junction resolvase	Boddy et al. 2001
<i>rad22</i>	52	3 ^h	Rad52 (32)	Rad51 accessory protein?	van den Bosch et al. 2002
<i>rti1</i>	42	0.8 ^h	Rad52 (32)	Rad51 accessory protein?	van den Bosch et al. 2002

Table 3 (continued)

Gene	Protein size (kDa)	Approx. extent of reduction by mutation	Putative <i>S. cerevisiae</i> ortholog (~% identity)	Inferred primary activity	Refs. for role in recombination
<i>swi4</i>	115	2–3	Msh3 (33)		Tornier et al. 2001
<i>pms1</i>	88	gc effects ⁱ	Pms1 (33)	Mismatch repair (MMR) ^j	Fleck et al. 1999
<i>msh2</i>	110	gc effects ⁱ	Msh2 (42)	Mismatch repair (MMR)	Fleck et al. 1999
<i>msh6</i>	141	gc effects ⁱ	Msh6 (39)	Mismatch repair (MMR)	Tornier et al. 2001
<i>swi10</i>	29	gc effects ⁱ	Rad10 (37)	Mismatch repair (NER) ^k	Fleck et al. 1999
<i>rad13</i>	126	gc effects ⁱ	Rad2 (35)	Mismatch repair (NER)	Kunz and Fleck 2001
<i>exo1</i>	64	gc effects ⁱ	Exo1 (29)	5' → 3' ds DNA exonuclease	Szankasi and Smith 1995

^a Not determined. Requirement for meiotic recombination is assumed, based on other phenotypes similar in *rad32* mutants (for *rad50* and *nbs1*) or in *mus81* mutants (for *eme1*)

^b Regions of 79 and 27 amino acids show ~22% and 37% identity, respectively

^c Also called *rhp51*

^d A region of 160 amino acids shows ~21% identity

^e Reduction affects crossovers (xo) only; gene conversions are modestly increased in frequency

^f The *rad54 rdlh54* double mutant has greatly reduced spore viability and is severely defective in DSB repair

^g Crossovers are reduced by factors of 10–90; gene conversions are reduced by a factor of <2

^h The *rad22 rti1* double mutant has greatly reduced spore viability (van den Bosch et al. 2002) and is defective in recombinational repair of meiotic DSBs (Young et al. 2004)

ⁱ Frequency of intragenic recombinants (gene convertants) is decreased or increased by factors of up to 35, but generally less, depending on the markers tested for recombination

^j Mismatch repair

^k Nucleotide excision repair

7.1

The MRN Complex Is Needed for Removing Rec12 from DSBs But Not for DSB Formation

The Mre11-Rad50-Nbs1 complex (MRN) is a widely conserved eukaryotic protein complex with both exo- and endonuclease activities and is essential for meiotic recombination in all tested organisms. Consistent with this conserved phenotype, *S. pombe rad32* (*MRE11* ortholog) and *rad50* mutants have strongly reduced spore viability and meiotic recombination and fail to repair meiotic DSBs (Tavassoli et al. 1995; Young et al. 2004). In contrast, *S. cerevisiae* MRN null mutants fail to make breaks (see Sect. 5.5 and Sect. 13).

The three components of the MRN complex have distinct roles. Rad50, an ATPase, is a member of the structural-maintenance-of-chromosomes (SMC) family of proteins, which have a long coiled-coil hairpin-like structure. This structure may allow Rad50 to co-ordinate events at the two sides of a meiotic DSB. The nuclease domain of Rad32 is required for processing DSBs; Rad50 appears to regulate this nuclease, as the *rad50S* (K81I) mutant accumulates Rec12-DNA complexes (Young et al. 2002; R. Hyppa, pers. comm.). The Nbs1 subunit is also believed to be regulatory.

S. pombe MRN nuclease-deficient mutants, like MRN null mutants, accumulate meiotic DSBs (Young et al. 2002, 2004; J. Farah, pers. comm.). Despite the 3' to 5' exonuclease polarity of the human and *S. cerevisiae* enzymes (Paull and Gellert 1998; Usui et al. 1998), it has been suggested that the MRN exonuclease is required for 5' end resection. However, *S. cerevisiae* MRN appears to cleave DNA ~10–40 nucleotides from the covalently linked Spo11 (Neale et al. 2005). *S. pombe* MRN nuclease mutants are also unable to remove Rec12 from the sites of DSBs (E. Hartsuiker, pers. comm.), and they can carry out meiotic recombination initiated by the *I-SceI* endonuclease (J. Farah, pers. comm.), which, unlike Rec12, does not remain covalently linked to the DNA. These data suggest that the sole nucleolytic role of MRN is to remove Rec12 from the ends of meiotic DSBs, with 5' resection being carried out by another enzyme. The responsible exonuclease is unknown. Exonuclease I has the appropriate specificity but seems to play little role other than mismatch correction in *S. pombe* meiotic recombination (Szankasi and Smith 1995; see Sect. 10).

7.2

Loading Strand-Exchange Proteins: Many Actors with Overlapping Roles

Strand-exchange proteins, bound to ss DNA, generate the joint molecule intermediates of recombination. However, these proteins alone are unable to compete with ss DNA binding (SSB) proteins for DNA. Consequently, from bacteriophages to humans, accessory proteins are needed to assist in their loading. *S. pombe* possesses several such accessory proteins: Rad22, Rti1, the

Rhp55-Rhp57 and Swi5-Sfr1 complexes, and possibly the Rlp1 protein. Rad22 and Rtl1 (also called Rad22B) appear to have redundant functions, but a suppressor mutation commonly found in *rad22* strains has complicated their analysis (Doe et al. 2004). The *S. pombe* Rhp55, Rhp57, and Rlp1 proteins are paralogs of the strand exchange protein Rad51 (also called Rhp51; Grishchuk and Kohli 2003). The Rhp55-Rhp57 and Swi5-Sfr1 complexes promote Rad51 and Dmc1 loading onto ss DNA (Haruta et al. 2006). Mutations in *rlp1*, *rhp55*, *rhp57* or the double *rhp55-rhp57* mutation have relatively mild effects on meiotic recombination frequencies and spore viability (Grishchuk and Kohli 2003). Mutations in *swi5* or *sfr1* also show a moderate reduction in meiotic recombination and slightly lowered spore viability (Young et al. 2004; unpublished data). In contrast to the single mutants, double mutants affecting both the Rhp55-Rhp57 and the Swi5-Sfr1 complexes have severe defects in spore viability and recombination, similar in magnitude to a *dmc1 rad51* double mutant (Ellermeier et al. 2004), suggesting that the two complexes possess redundant functions. However, the exact roles of these proteins, and the distinctions between them, remain unclear.

8

Strand Invasion and Partner Choice

By analogy with recombination in other organisms, the action of Rad22, Rtl1, Rlp1, Rhp55-57 and Swi5-Sfr1 is believed to produce DNA ends with 3' overhangs coated in strand exchange proteins. Through the process of strand invasion, this nucleoprotein complex generates joint molecules between homologous DNA duplexes that can then be processed to give recombinants.

8.1

The Dmc1 and Rad51 Strand Exchange Proteins: Finding a Homologous Partner for Recombination

The archetypal DNA strand exchange protein is *Escherichia coli* RecA, which is loaded onto ss DNA by the RecBCD or the RecFOR proteins to form a nucleoprotein filament capable of strand exchange (see Prévost, this book). The filament pairs with the complementary strand of a homologous duplex, displacing the other strand to form a displacement loop (D-loop). This joint molecule is held together by a region of hybrid DNA, having one strand from each parent. Finally, RecA cycles off the DNA after hydrolysis of ATP.

S. pombe possesses two RecA structural and functional homologs – Rad51, which is expressed in all cells of all studied eukaryotes, and Dmc1, which is meiosis-specific but absent from some species. Mutations in *rad51* and *dmc1* have quite distinct effects on meiotic recombination. Although both mutations show an approximately 5-fold reduction in crossing-over, the ef-

fect of the *rad51* mutation on gene conversion (non-reciprocal recombination) is much stronger. The spore viability of a *rad51* mutant is very low, while that of a *dmc1* mutant is close to the wild-type level (Grishchuk and Kohli 2003). The relative spore viabilities can be explained by the observation that in *dmc1* mutants meiotic DSBs are repaired, but in *rad51* mutants, they are not repaired (Young et al. 2004; see Sect. 13). The recombination and spore viability phenotypes of the double mutant are much more severe than those of the single mutants, suggesting some redundancy in the function of the two proteins (Grishchuk and Kohli 2003). Double mutant analyses suggest that Dmc1, Swi5, and Sfr1 function in one branch of a pathway and Rhp55 and Rhp57 in another (Ellermeier et al. 2004; Fig. 1). In addition, the Mcp7-Meu13 complex, which is homologous to *S. cerevisiae* Mnd1-Hop2, also appears to act specifically with Dmc1. Mutations in either *mcp7* or *meu13* have mild spore-viability defects and substantial recombination defects (Saito et al. 2004), but the exact function of this complex is unclear. Further studies are needed to elucidate these aspects of *S. pombe* meiotic recombination.

8.2

The Rhp54 and Rdh54 Proteins: Enabling Strand Exchange in a Chromatin Context?

S. pombe Rhp54 and its paralog Rdh54 are members of the Swi2 (Snf2) family of proteins, many of which remodel chromatin. Rhp54 is expressed both mitotically and meiotically, while Rdh54 is meiosis-specific (Catlett and Forsburg 2003). *rhp54* and *rdh54* mutants show mild defects in recombination, spore viability, and meiotic DSB repair, but the double mutant is severely defective. The *S. cerevisiae* orthologs of *rhp54* and *rdh54* appear to alter chromatin structure to facilitate Rad51 and Dmc1 strand exchange (Heyer et al. 2006). The *S. pombe* proteins may act similarly, since Rhp54 interacts with Rad51, and Rdh54 interacts with both Rad51 and Dmc1 (Catlett and Forsburg 2003). Mutations in *S. pombe rdh54* have meiotic phenotypes similar to those of *dmc1* mutations (high spore viability and successful repair of DSBs but reduced recombination), suggesting that these two meiosis-specific proteins may act in the same pathway.

8.3

Intersister vs. Interhomolog Recombination: Any Partner Will Do?

In meiosis there are almost always three homologous DNA targets with which a recombinogenic DNA end can interact – the sister chromatid or either of the two chromatids of the homolog. Interhomolog recombination might be expected to be favored over intersister recombination because meiotic recombination must produce crossovers between homologs for their proper segregation (see Mehrotra, Hawley and McKim, this SERIES). Results from

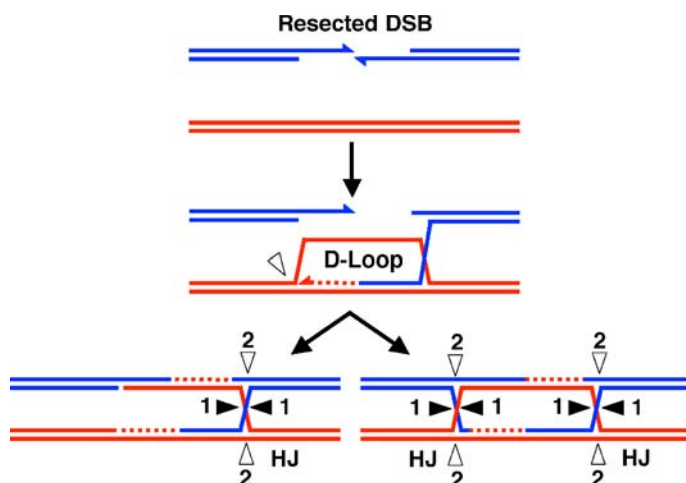


Fig. 3 A scheme that produces either crossover or non-crossover recombinants from single or double Holliday junctions. Resection of a DSB produces two 3' single strand overhangs, one of which invades a homologous duplex, producing a D-loop. A single HJ results if this D-loop is cut before second end capture (*left*). A double HJ results if the D-loop remains uncut before second end capture (*right*). In both cases resolution of the HJ(s) results in crossover or non-crossover products, depending on the strands cleaved

S. cerevisiae support this idea (see Sect. 12). However, genetic studies and physical analysis of joint molecules in *S. pombe* have demonstrated that intersister recombination does occur and is actually more frequent than interhomolog recombination (Cromie et al. 2006). How this is reconciled with the necessity of producing interhomolog crossovers is discussed in Sect. 12. Nevertheless, *S. pombe* does appear to have mechanisms that specifically promote interhomolog recombination (see Sect. 3). As discussed above, *dmc1* mutants have reduced recombination frequencies, but they repair meiotic DSBs and have high spore viability. This suggests that Dmc1 promotes the repair of DSBs using homologs, but, if Dmc1 is absent, breaks are repaired using sister chromatids. The distinct phenotypes of *S. cerevisiae* vs. *S. pombe dmc1* mutants may result from a mechanism, present in *S. cerevisiae* but not *S. pombe*, that inhibits intersister recombination (see Sect. 12 and 13).

9

Joint Molecule Resolution

In the current canonical model of meiotic crossing over (Szostak et al. 1983; Sun et al. 1989), after strand invasion and D-loop formation the second end of the initiating DSB anneals to the D-loop (Fig. 3). Branch migration at each

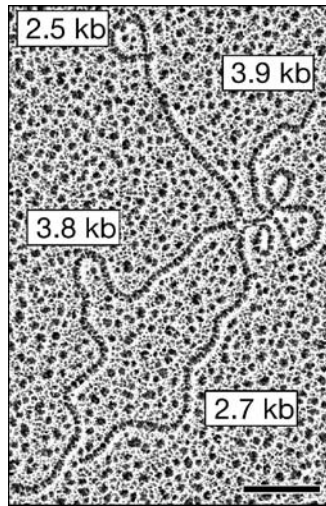


Fig. 4 Single Holliday junction intermediates in *S. pombe* meiotic recombination. DNA from meiotic *mus81* mutant cells was separated by two-dimensional gel electrophoresis, and DNA in the joint molecule region was visualized with an electron microscope. The junction of the four DNA duplex segments is splayed out in a “traffic circle” due to formamide in the spreading mixture. Bar indicates 0.2 μm . From Cromie et al. (2006)

side of the D-loop then generates two Holliday junctions (double HJs, Fig. 3 right) connecting the homologous duplexes. Cleavage and rejoining of appropriate pairs of strands in the two HJs can then generate a crossover.

9.1

Single Holliday Junctions: An Unexpected Recombination Intermediate

Electron microscope studies of meiotic joint molecules in *S. pombe* reveal DNA structures different from those predicted by the current canonical model and previously observed in *S. cerevisiae* (Cromie et al. 2006). Instead of double HJs, the majority of molecules contain single HJs (Fig. 4). Two dimensional gel electrophoretic analyses are also consistent with a majority of single HJs. The single HJ and double HJ mechanisms may differ only by the timing of cleavage of the strand forming the D-loop: cleavage before second end capture produces a single HJ (Fig. 3, left), whereas cleavage after second end capture allows formation of a double HJ (Fig. 3, right).

9.2

Mus81-Eme1: The Meiotic Holliday Junction Resolvase of *S. pombe*

To generate crossovers from joint molecules, the HJs must be cleaved and the broken strands ligated. The *S. pombe* Mus81-Eme1 endonuclease can re-

solve HJs and closely related structures (Boddy et al. 2001). *mus81* and *eme1* mutants have, as far as tested, indistinguishable phenotypes which are those expected of nuclear HJ resolvase mutants. Most notably, HJs accumulate in *mus81* mutant meiosis (Cromie et al. 2006). Physical and genetic assays show that meiotic crossovers, which are expected to depend on an HJ resolvase, are greatly reduced in *mus81* mutants, whereas genetic assays show that gene conversions without crossovers, which are not necessarily resolvase-dependent, are essentially unaffected (Osman et al. 2003; Smith et al. 2003). In *mus81* mutants the asci are aberrantly shaped, spore viability is very low, and there is a single mass of apparently entangled DNA. These phenotypes are indicative of chromosome segregation failure, as expected from the chromosomes remaining held together by unresolved HJs. The phenotypes of *mus81* mutants can be relieved by expression of a bacterial HJ resolvase. As expected, in meiosis *rec12Δ* is epistatic to *mus81Δ* and *eme1Δ*, indicating that Mus81-Eme1 is required for meiotic DSB repair. Amino acid substitutions affecting the Mus81 nuclease active site have a phenotype indistinguishable from that of a complete deletion. Collectively, these observations indicate that the complex's role in crossing-over is endonucleolytic resolution (Boddy et al. 2001; Osman et al. 2003; Smith et al. 2003).

The archetypal HJ resolvase, *E. coli* RuvC, cleaves intact HJs by symmetrical cleavages of two strands; the cleaved product is a good substrate for DNA ligase. Mus81-Eme1 purified from *S. pombe* and human cells cleaves intact HJs, although not symmetrically. All reported preparations of Mus81-Eme1 show higher activity on three-strand junctions than on intact HJs, but nicked HJs are preferred over the 3-strand junctions, with cleavage occurring at the site on the HJ strand opposite the nick (Doe et al. 2002; Gaillard et al. 2003; Osman et al. 2003). Mus81-Eme1 may readily cleave 3-strand junctions because they resemble nicked HJs. Osman et al. (2003) have proposed a model of recombination involving Mus81-Eme1 cleavage of nicked HJs. However, Mus81-Eme1 purified from *S. pombe* and human cells slowly cleaves one strand of an intact HJ and then rapidly cleaves the nicked product (Gaillard et al. 2003); this may be the relevant *in vivo* activity, perhaps stimulated by other factors. Asymmetric HJ cleavage would prevent immediate ligation of the cut strands, but repair DNA synthesis and single-strand flap-processing would allow the required ligation.

10

Mismatch Correction

Gene conversion is a form of recombination involving the non-reciprocal transfer of sequence information between homologous DNA sequences. It is observed as heterozygous marker segregation other than the normal Mendelian 2:2 among the four spores in an ascus and is often produced as

an outcome of mismatch correction. Hybrid DNA is generated between homologous loci undergoing strand exchange, and, if the loci are non-identical in sequence, the hybrid DNA will contain mismatches. Repair of such mismatches can lead to gene conversion, seen as 3:1 segregation among the four spores. Unrepaired mismatches segregate at the first round of DNA replication after meiosis and, thus, can be identified as post-meiotic segregation (PMS) events, detectable as a sectorized colony arising from one spore.

In *S. pombe*, both mismatch repair (MMR) and nucleotide excision repair (NER) function in the repair of mismatches arising during meiotic recombination. Presumably, after mismatch recognition by MMR proteins and strand incision by unknown factors, exonuclease I removes part of a strand with one of the mismatched nucleotides and repair DNA synthesis restores complementarity. MMR appears to repair efficiently small insertion or deletion loops and all base mismatches other than C/C. The C/C mismatch is repaired, but inefficiently, by the NER system, which can also repair other base mismatches in the absence of the MMR pathway (Fleck et al. 1999). The MMR and NER gene products demonstrably required for these processes are shown in Table 3. In meiotic crosses involving the *ade6-M26* hotspot, essentially all mismatch correction (identified as 3:1 marker segregation) is abolished in the absence of both the MMR and NER pathways, and only PMS events are seen, at elevated frequency. One class of PMS event has hybrid DNA in one spore (~70% of all asci with non-2:2 segregation), and another class has hybrid DNA in two spores (~30%). The first class may reflect a mismatch in the region resected adjacent to a meiotic DSB (asymmetric heteroduplex), and the second class may reflect a mismatch in a more distant region where branch migration formed an HJ (symmetric heteroduplex). Thus, both symmetric and asymmetric hybrid DNA forms appear to be frequent in *S. pombe* meiosis.

The fission yeast *swi4* gene encodes a homolog of the budding yeast MMR protein Msh3. However, rather than a meiotic MMR defect, mutations in *swi4* exhibit a mild deficiency in both intragenic and intergenic recombination. The function of Swi4 in meiotic recombination is unclear.

11

Relation of Gene Conversion and Crossing-over

Gene conversion indicates hybrid DNA at a marked locus and, thus, strand exchange. Markers flanking this locus may or may not undergo crossing-over. Until recently, it was generally believed that there was a single pathway of homologous recombination with an HJ intermediate containing hybrid DNA. At the HJ resolution stage, if the pairs of complementary strands cleaved in the HJs are chosen at random, crossovers and non-crossovers would be equally frequent (Fig. 3). Recent work in both prokaryotes and eukaryotes

has thrown doubt on this model and has suggested that crossovers and non-crossovers are generated by different pathways (Cromie and Leach 2000; Allers and Lichten 2001). The mechanism that generates non-crossovers is unclear but may involve sequential DNA synthesis, DNA unwinding, and annealing, termed “synthesis-dependent strand annealing” (SDSA; see Haber, this book; Lankenau, 2007, this SERIES). Gene conversion could occur in the hybrid DNA envisaged as a part of the SDSA model.

In *S. pombe*, crossovers accompany gene conversions $\sim 75\%$ of the time rather than 50% , as predicted by the random HJ resolution model, or $\sim 40\%$, as observed in *S. cerevisiae* (Grimm et al. 1994; Cromie et al. 2005). In addition, as noted in Sect. 9.2, *mus81* resolvase mutations have very little effect on the frequency of gene conversions that lack an associated crossover (Osman et al. 2003; Smith et al. 2003). This suggests that these non-crossovers do not result from HJ resolution. Therefore, it appears that in *S. pombe* crossovers result from HJ resolution and non-crossovers from a second mechanism, such as SDSA, although HJ resolution may contribute to some non-crossover events.

12

Species-specific Strategies for Ensuring, With or Without Interference, the Crossovers Required for Chromosome Segregation

Meiotic crossovers generate new combinations of alleles that increase genetic diversity in the population, and in most organisms, crossovers also aid the correct segregation of homologs at the first meiotic division. Intersister recombination achieves neither of these aims. Why then does *S. pombe* show a bias towards intersister events, while *S. cerevisiae* shows a bias towards interhomolog events (Cromie et al. 2006)? Similarly, why is crossover interference, which reduces the probability of crossovers occurring close together, present in *S. cerevisiae* and not in *S. pombe* (Munz 1994)?

The bias to interhomolog recombination seen in *S. cerevisiae* appears to result from a barrier to intersister recombination events (Niu et al. 2005), i.e., it is a form of regulation of recombination, as is crossover interference. Some of the 16 chromosomes of *S. cerevisiae* are very small, as short as 230 kb, and all are smaller than the smallest of *S. pombe* (3500 kb). If the total number of crossovers in *S. cerevisiae* were distributed randomly across the DNA (i.e., if there were no interference), then these small chromosomes would receive no crossover $\sim 10\%$ of the time and would frequently missegregate. Interference may ensure that crossovers are distributed so that small chromosomes always receive at least one. Interhomolog bias may be a further adaptation to ensure enough interhomolog crossovers on small chromosomes without increasing the number of DSBs.

In contrast to *S. cerevisiae*, *S. pombe* has only three, large chromosomes. Random (Poisson) distribution of crossovers suffices to ensure that each chromosome receives at least one interhomolog crossover, since there is an average of 10, 15, and 20 interhomolog crossovers per meiotic cell (Munz et al. 1989). The average number of crossovers on the smallest chromosome is high enough to prevent missegregation without needing to regulate recombination through crossover interference (Munz et al. 1989) or a barrier to intersister recombination. In this view, *S. pombe* uses a meiotic recombination system “pared down” to its fundamentals. The obvious success of this scheme raises the question of why other organisms regulate crossovers rather than alter the size and number of chromosomes or increase the number of DSBs so that unregulated recombination would allow successful chromosome segregation.

13

Differences Between *S. pombe* and *S. cerevisiae* Meiotic Recombination: A Reprise

At all steps of meiotic recombination, significant differences are seen between *S. pombe* and *S. cerevisiae* – in the production and processing of DSBs, in the loading of strand exchange proteins, in choice of partner for the strand exchange reaction, in the structure of joint molecules, and in the processing of joint molecules into recombinant products. These differences may account for the differences, noted in Sect. 1, 3, and 11 and below, in the occurrence of interference, the frequency of ectopic recombination, and the frequency of crossovers associated with gene conversion.

Several components for the formation and processing of meiotic DSBs differ markedly in *S. pombe* and *S. cerevisiae*. Each species has proteins essential for DSB formation that have no obvious ortholog in the other species (Table 2). Furthermore, the Rec8 cohesin subunit is required for DSB formation in *S. pombe* but not in *S. cerevisiae*; it is required for DSB repair in *S. cerevisiae* but perhaps not in *S. pombe* (Klein et al. 1999; Ellermeier and Smith 2005). Conversely, the MRN nuclease complex is required for DSB formation in *S. cerevisiae* but not in *S. pombe*; it is required for DSB repair in both species (Cao et al. 1990; Young et al. 2004). Presumably, Rec8 and MRN are required indirectly and differentially in the two species for the assembly of the Rec12 (Spo11) complex at sites of DSB formation. DSB repair in both species may require the MRN nuclease to remove Rec12 (Spo11) linked to the DNA (see Sect. 7.1). The role of Rec8 in DSB repair in *S. cerevisiae* is unclear.

There are several differences between *S. cerevisiae* and *S. pombe* relating to Rad51 accessory proteins. First, the function of the *S. cerevisiae* Rad52 protein appears to be carried out by two partially redundant Rad52 homologs in *S. pombe*. Interestingly, *rad52*^{-/-} knockout mice are viable and fertile (Rijkers et al. 1998), suggesting that mammals may be more similar in this regard

to *S. pombe* than to *S. cerevisiae*. Second, mutations in the *rad55*, *rad57* and *rdh54* genes have very severe recombination defects in *S. cerevisiae*, but the *S. pombe* ortholog mutants have only mild defects (Petes et al. 1991; Catlett and Forsburg 2003; Grishchuk and Kohli 2003). Third, the *S. pombe* Swi5-Sfr1 complex appears to function in both mitosis and meiosis, unlike the *S. cerevisiae* meiosis-specific Sae3-Mei5 ortholog. This is consistent with the ability of Swi5-Sfr1 to promote both Rad51 and Dmc1 activity (Haruta et al. 2006), whereas Sae3-Mei5 is believed to interact only with Dmc1 (Hayase et al. 2004). Fourth, there appears to be no Rlp1 ortholog in *S. cerevisiae*, but mammals have an ortholog, Xrcc2 (Khasanov et al. 2004).

The mechanics of strand invasion are not known to differ between *S. pombe* and *S. cerevisiae*. However, significant differences are seen in the choice of DNA used as the target of strand invasion; i.e., in *S. pombe* intersister events are preferred, while in *S. cerevisiae* interhomolog events predominate (see Sect. 8.3 and 12). These differences appear to be related to differences in the phenotypes of *dmc1* mutants. *S. pombe dmc1* mutants successfully repair meiotic DSBs, presumably using sister chromatids. In contrast, in *dmc1* mutants of *S. cerevisiae* strain SK1, DSBs remain unrepaired (Bishop et al. 1992), apparently due to a barrier to intersister recombination that involves the Hop1, Mek1 and Red1 proteins (Niu et al. 2005). The apparent absence of this barrier in *S. pombe* may explain why intersister events are more frequent in *S. pombe* than in *S. cerevisiae*.

The structures of recombination joint molecules in *S. pombe* and *S. cerevisiae* are distinctly different. As discussed in Sect. 9.1, most recombination joint molecules in *S. pombe* contain single HJs rather than the double HJs seen in most *S. cerevisiae* joint molecules and predicted by a current recombination model (Szostak et al. 1983). A variation on this model can account for either a single HJ or a double HJ arising from a D-loop and producing recombinants (Fig. 3).

In *S. pombe* the Mus81-Eme1 complex appears to be the only meiotic nuclear HJ resolvase. However, despite the wide conservation of these two proteins among eukaryotes and the similar mitotic phenotypes of the mutants, the importance of these proteins in meiosis appears to vary among organisms. In *S. cerevisiae* crossovers are only mildly reduced by *mus81* or *mms4* (*eme1*) mutations, and in mice *mus81* mutants are viable and fertile (de los Santos et al. 2003; McPherson et al. 2004). *S. cerevisiae* and mice may have a second crossover pathway that requires the Msh4 and Msh5 proteins, which are apparently absent from *S. pombe*. Interestingly, *mus81*-dependent crossovers in *S. cerevisiae*, like those of *S. pombe*, are not subject to crossover interference (de los Santos et al. 2003).

The similarities of meiotic recombination in *S. pombe* and *S. cerevisiae* indicate that the basic process of DSB formation and repair is widely conserved and perhaps universal. But the multiple differences suggest that the regulation of meiotic recombination is variable among species and may have arisen

independently during evolution. Further understanding of these similarities and differences may provide insight into how meiotic recombination arose, presumably from a mitotic precursor.

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Nuclear Movement Enforcing Chromosome Alignment in Fission Yeast—Meiosis Without Homolog Synapsis

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Abstract Pairing of homologous chromosomes is a prerequisite for meiotic recombination. In most eukaryotic organisms, a synaptonemal complex is formed between homologous chromosomes along their entire lengths to stabilize pairing. Asynaptic organisms in which the canonical synaptonemal complex is not formed are still able to pair homologous chromosomes in a different way. In the fission yeast *Schizosaccharomyces pombe*, telomere clustering and oscillatory nuclear movement persist for the entire meiotic prophase. This characteristic chromosome arrangement promotes pairing of homologous chromosomes and may compensate for the lack of homolog synapsis. Here we review recent progress in elucidating the dynamics of homologous pairing, as well as the molecular mechanisms involved in the regulation of telomere clustering and nuclear movement in *S. pombe*.

Abbreviations

FISH fluorescence in situ hybridization
GFP green fluorescent protein
KASH Klarsicht-ANC1-SYNE1 homology domain
LE linear elements
SC synaptonemal complex
SPB spindle-pole body
SUN Sad1-UNC84 homology domain

1

Introduction

Meiosis is a process that generates haploid gametes from a diploid parental cell through one cycle of DNA synthesis, followed by two consecutive chromosome segregations. Pairing and disjunction of homologous chromosomes are unique features of the first meiotic division (meiosis I), in which homologous chromosomes locate each other, establish physical association via crossing-over recombination, and segregate to the opposite poles of the spindle (reviewed in Gerton and Hawley 2005). Spatial alignment of homologous chromosomes is the first step in this process. During meiotic prophase, a highly

conserved phenomenon among eukaryotes is the formation of a “chromosome bouquet”—a polarized chromosome arrangement with all the telomeres clustered in a confined area on the nuclear envelope (reviewed in Scherthan 2001). Accompanying bouquet formation, chromatin compaction and chromosome movement usually occur. The ultimate form of homologous association is synapsis, in which the entire lengths of homologous chromosomes are connected by the synaptonemal complex (SC), a tripartite proteinaceous structure. The function of SC is not well understood. It is assumed that the function of SC is to stabilize the homologous interactions and protect chiasmata (inter-homologous physical links generated from crossing-over recombination). Chiasmata are essential for faithful disjunction of homologous chromosomes at meiosis I, providing a force balance between bipolar spindle attachments.

While SC is found in a wide range of eukaryotes, there are a few organisms in which chiasmata formation is not accompanied by a SC. This is the case in the fission yeast *Schizosaccharomyces pombe* and in the ciliate protist *Tetrahymena thermophila*. Interestingly, instead of forming SC, these organisms have evolved specialized mechanisms that promote homologous interactions. In *Tetrahymena*, the meiotic prophase nucleus is strikingly elongated to form a thread-like crescent with telomeres clustered at one end, which may function to promote the parallel arrangement and juxtaposition of homologous chromosomes (Loidl and Scherthan 2004). Similarly, in *S. pombe*, the nucleus is elongated during meiotic prophase and all telomeres are tightly clustered. The telomere-clustered nucleus moves dramatically during meiotic prophase (Chikashige et al. 1994). These nuclear movements with telomere clustering are essential for achievement of homologous pairing in *S. pombe* (Ding et al. 2004). In this organism, instead of the SC, structures known as linear elements (LE) form during meiotic prophase, although the function of LE is not clear (for further information on LE, see the review by Loidl 2006 and references therein). In addition to the absence of SC, crossover interference, a widely conserved phenomenon in which one crossover somehow suppresses the occurrence of other crossovers nearby (Hillers 2004) was not found in *S. pombe* (Munz 1994). However, since evidence has shown that the SC is actually not required for interference in budding yeast and *Drosophila* (see the review by Bishop and Zickler 2004), the reason for lacking interference in *S. pombe* remains to be explored. Here we focus on *S. pombe* to summarize recent advances in functional analyses of telomere clustering and nuclear movement in relation to homologous chromosome pairing, including the underlying molecular mechanisms.

2

Alignment of Homologous Chromosomes

2.1

Meiosis in *S. pombe*

S. pombe cells are maintained in the haploid state during the vegetative growth stage of the lifecycle. Upon nitrogen starvation, haploid cells of opposite mating types conjugate, forming a diploid nucleus through karyogamy. The process of meiosis normally begins immediately without any intervening mitotic cycles (zygotic meiosis). Two rounds of meiotic chromosome segregation, involving the separation of homologous chromosomes at meiosis I and sister chromatids at meiosis II, result in the formation of four haploid spores (Fig. 1). When under the selective pressure with some auxotrophic markers, cells can also be maintained in the diploid state; the meiosis start in a diploid cell is called “azygotic meiosis”.

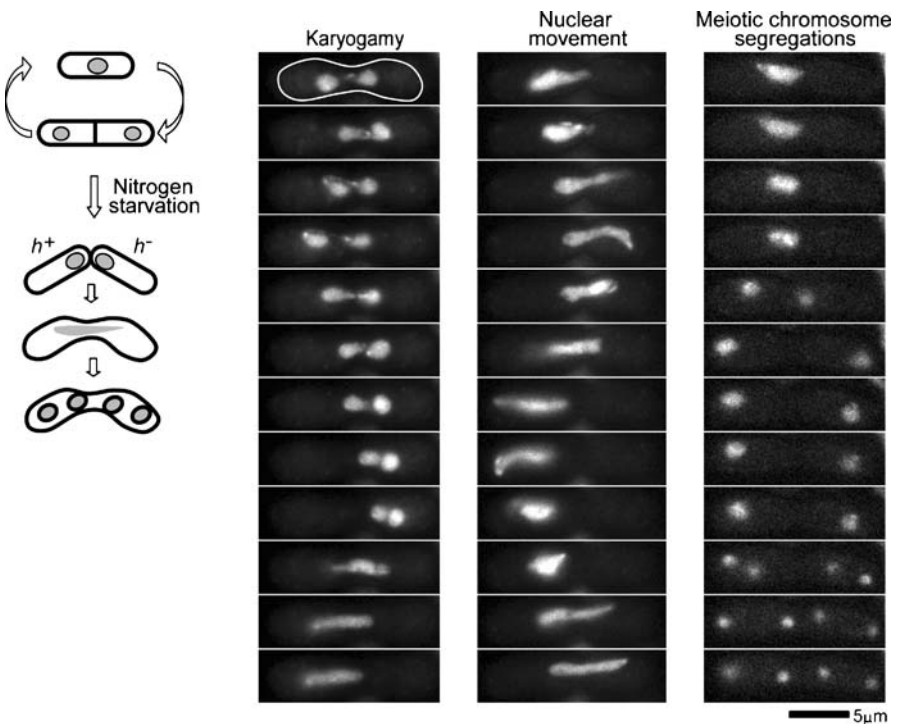


Fig. 1 Meiosis in *S. pombe*. Upon nitrogen starvation, haploid cells of the opposite mating type, h^+ and h^- , conjugate and enter zygotic meiosis. Time lapse images of nuclei during karyogamy (2 min interval), horsetail movement (1 min interval) and chromosome segregations (10 min interval) are shown. DNA was stained with Hoechst33342

Pairing and recombination of homologous chromosomes occur during meiotic prophase and are important for subsequent chromosome segregation. Meiotic prophase in *S. pombe* is characterized by an elongated nucleus called a “horsetail” nucleus because of its shape. The horsetail period starts immediately after nuclear fusion, and continues for a few hours preceding metaphase of the first meiotic division (Fig. 1). The horsetail period also involves premeiotic DNA synthesis. Two characteristic cytological features in meiotic prophase are telomere clustering and nuclear movement. Telomere clustering starts in response to mating pheromone signaling prior to karyogamy (Chikashige et al. 1994, 1997). During telomere clustering, all of the telomeres originally dispersed on the periphery of the nucleus move to a position beneath the spindle pole body (SPB; a fungal microtubule organization center equivalent to the centrosome in animals) and form a tight cluster, while centromeres anchored beneath the SPB in mitotic interphase detach (Fig. 2A). The telomere clusters from each haploid gamete fuse during karyogamy. After karyogamy, the diploid nucleus elongates about three times in length ($\sim 7 \mu\text{m}$) and initiates oscillation from one end to the other in the cell, forming the horsetail nucleus. The horsetail nucleus continuously moves during the entire period of meiotic prophase. This movement is driven by microtubules emanating from the SPB (Ding et al. 1998); telomeres clustered beneath the SPB always locate at the leading edge of the moving nucleus. Telomere clustering and nuclear movement are also observed in azygotic meiosis of diploid cells.

2.2

Contribution of Telomere Clustering and Nuclear Movement to Homologous Chromosome Alignment

Homologous pairing in *S. pombe* has been extensively studied using both FISH and LacO/LacI-GFP labeling methods¹ (Ding et al. 2004; Nabeshima et al. 2001; Niwa et al. 2000; Saito et al. 2005; Scherthan et al. 1994; Shimanuki et al. 1997). In zygotic meiosis, haploid nuclei fuse with the telomere-SPB cluster at the leading edge, immediately followed by the telomere-led horsetail nuclear movements. Nuclear movements align homologous chromosomes in a roughly parallel orientation (Fig. 2A). Direct observation of homologous pairing in individual living cells revealed several unique features of homologous interaction in *S. pombe*. (1) Interaction between homologous loci is dynamic, and homologous loci repeatedly associate and dissociate. (2) As a result of telomere clustering, the chromosome loci proximal to the telomere show higher associa-

¹ Homologous loci were fluorescently labeled by FISH using specific DNA probes in fixed specimens, and examined for the paired or unpaired signals. To follow pairing of homologous loci in living cells, repeats of bacterial lac operator sequences (LacO) were integrated into a specific chromosome locus, and visualized by expressing GFP-fused Lac repressor (Lac I-GFP) which specifically binds to lacO repeats.

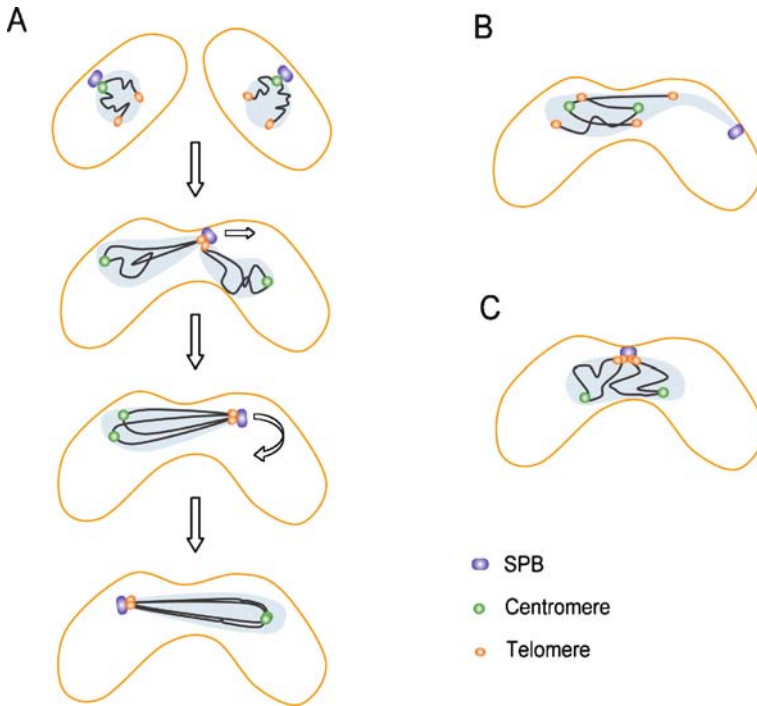


Fig. 2 Schematic view of the behavior of homologous chromosomes during meiotic prophase in wild-type cells (**A**), in mutant cells defective in telomere clustering (**B**), and in mutant cells defective in SPB integrity or motor proteins (**C**). Before entering meiotic prophase, centromeres form a cluster beneath the SPB, and telomeres are scattered along the nuclear envelope. In a conjugated zygote, telomeres form a cluster close to the SPB and centromeres become separated from the SPB. Homologous chromosomes that are in opposite directions during karyogamy are aligned to the same direction as a result of SPB/telomere-led nuclear movement. The nuclear movement and homologous recombination result in a further reduction in the spatial distance between homologous chromosomes, thereby facilitating completion of the pairing process. In mutant cells defective in telomere clustering, the SPB moves without accompanying chromosomes and thus fails to align the homologs (**B**). In mutant cells defective in SPB integrity or motor proteins, the oscillatory nuclear movement does not occur; this also causes failure of homologous chromosome alignment and pairing (**C**)

tion frequencies than those which are distal to the telomere. (3) The frequency of homologous interaction increases with the progression of meiotic prophase. This increase in homologous association requires homologous recombination. However, no instances of 100% association were found for any of the observed chromosome loci at any time in meiotic prophase. Thus, in contrast to synaptic meiosis, a stable association that links homologous interactions side-by-side along the entire length of chromosome is not present in an asynaptic organism like *S. pombe* (Ding et al. 2004).

In situations where telomere clustering or nuclear movements are compromised, homologous recombination is inhibited (Chikashige et al. 2006; Cooper et al. 1998; Davis and Smith 2006; Miki et al. 2002; Niccoli et al. 2004; Niwa et al. 2000; Saito et al. 2005; Shimanuki et al. 1997; Yamamoto et al. 1999). Observations revealed that a pair of homologous loci cannot get closer during the meiotic prophase in such mutants (Ding et al. 2004), demonstrating that both telomere clustering and nuclear movement are necessary to bring homologous loci together and facilitate their subsequent association (Fig. 2B,C). Recombination frequency in these mutants is usually 10 to 30% of that of wild-type cells. This decrease suggests that effective spatial homologous alignment by telomere clustering and nuclear movement can promote efficient homologous recombination. Moreover, an elevated frequency of ectopic (non-allelic) recombination is found in mutants impaired in telomere clustering and nuclear movement, indicating that chromosome alignment is necessary for restricting ectopic recombination (Davis and Smith 2006; Niwa et al. 2000; Saito et al. 2005). Therefore, persistence of telomere clustering and nuclear oscillation during the entire meiotic prophase may be a mechanism developed in this asynaptic organism to compensate for the lack of SC.

However, it should be noted that recombination can promote the alignment of homologous chromosome loci. Homologous association is affected in recombination mutants (Ding et al. 2004; Molnar et al. 2003; Nabeshima et al. 2001). In the absence of recombination, homologous interactions at chromosome arms were transient as the spatial distance between roughly aligned homologous chromosomes could not be decreased further (Ding et al. 2004). The underlying mechanism of recombination in promoting homologous chromosome pairing is not clear. It is possible that reciprocal recombination (crossing over) and resulting chiasmata provides a stable physical link and converging homologous chromosomes. Interestingly, the association between homologous centromeres is normal in the absence of recombination, suggesting a role for centromere heterochromatin in recombination independent homologous pairing (Ding et al. 2004). In the absence of meiotic recombination, random segregation of homologous chromosome at meiosis I is expected. However, the observed segregation is better than randomness in several recombination deficient mutants (Molnar et al. 2001; Davis and Smith 2003). The recombination-independent centromere pairing may contribute to improvement of fidelity in achiasmate chromosome segregation. Such recombination-independent mechanisms for centromere pairing remain to be elucidated.

2.3

Chromosome Architecture in the Alignment of Homologous Chromosomes

In most organisms, chromosome compaction increases in conjunction with the formation of the SC. Meiotic cohesins, as major components of the chro-

mosome axis, play critical roles in the assembly of SC (Revenkova and Jessberger 2006). In *S. pombe* meiosis, the Rec8-containing meiotic cohesin complex replaces the Rad21-containing mitotic cohesion complex, and this replacement is important for the meiosis-specific regulation of sister chromatid cohesion, recruitment of recombination proteins, and for meiosis-specific kinetochore orientation (see Tanaka and Watanabe, this series) (Fig. 3A). Rec8 also affects chromosome architecture. In cells lacking Rec8, chromosomes are under-compacted as if lacking a backbone, and only the leading edge of the nucleus oscillates; a major mass of chromosomes does not follow the movement (Ding et al. 2006) (Fig. 3B). In contrast, cells lacking Pds5 (cohesin-associated protein) produce a short over-compacted axis of chromosomes (Ding et al. 2006) (Fig. 3B). In both of these mutants, spatial alignment of homologous chromosomes is largely inhibited (Ding and Hiraoka, unpublished results). The reduction of homologous association in Rec8-deficient mutants is more severe than that observed in recombination mutants (*rec12⁻* mutant, for example). Thus, Rec8 may contribute to homologous pairing through both recruitment of recombination proteins and establishment of meiosis-specific chromosome architecture. On the other hand, chromosome compaction is normal even in the absence of LE (Ding et al. 2006), indicating that meiotic cohesins, rather than LE, play a major role in formation of chromosome architecture in this organism.

3

Regulation of Telomere Clustering

It has been demonstrated that telomere clustering is an important initial step for homologous chromosome pairing and recombination during asynaptic meiosis in *S. pombe*. Here we summarize mechanisms for telomere clustering.

3.1

Mating Pheromone, MAP Kinase and Mei2

Telomere clustering occurs during conjugation in response to the mating pheromone (Chikashige et al. 1997). In *S. pombe*, pheromone signal transduction is mediated through the pheromone-responsive MAP kinase (MAPK) cascade, in which *byr2* and *spk1* genes encode MAPKKK, MAPKK and MAPK, respectively. Expression of Byr1^{DD}, a constitutively active form of MAPKK for Spk1 MAPK, induces ectopic meiosis and telomere clustering in haploid cells in the absence of a mating pheromone response (Yamamoto et al. 2004).

Telomere clustering also requires Mei2, a key regulator of meiosis in fission yeast. Mei2 is repressed by Pat1 kinase in vegetative growing cells. Suppression of Pat1 kinase or constitutive expression of Mei2 both induces telomere clustering and meiosis in haploid cells (Chikashige et al. 2004; Watanabe et al.

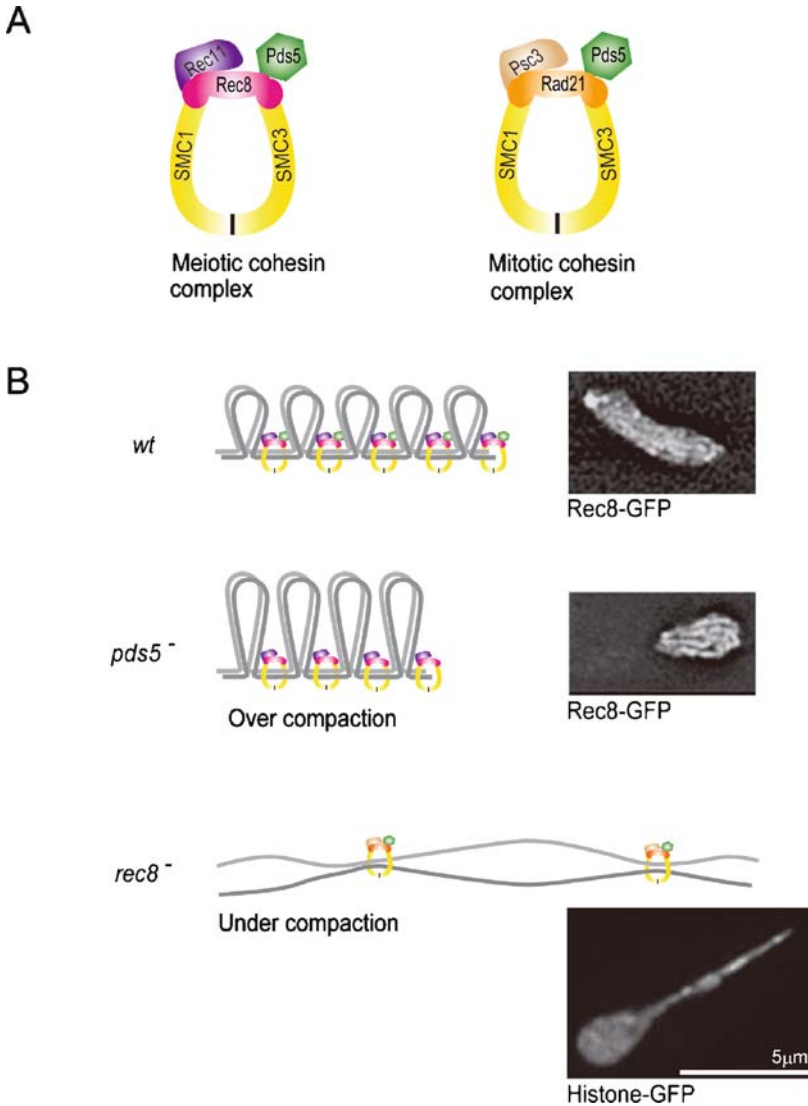


Fig. 3 Cohesin complex and chromosome structure in meiotic prophase. **A** A model of meiotic and mitotic cohesin complex in *S. pombe*. In meiotic prophase, Rec8 and Rec11 replace Rad21 and Psc3 on the arm region of chromosomes. **B** A model for meiotic cohesins in promoting the compaction of meiotic prophase chromosomes. Meiotic cohesin complexes replace mitotic cohesin complexes during meiotic prophase and compact the chromosome. Meiotic cohesin complexes act as a foothold for looping chromatin along the axis. The cohesin-axis is visualized using Rec8-GFP fusion protein in live cells. In the absence of Pds5, the density of meiotic cohesin complex binding sites is reduced and thus results in larger loops between each binding site and a shorter longitudinal axis. In the absence of Rec8, the meiotic cohesin complex is absent from chromosomes; the meiotic cohesin-axis and chromatin loops are not formed and the chromatin is under-compacted

1997; Yamamoto et al. 2004). However, Pat1 kinase inactivation does not induce completely normal meiosis even in diploid cells. Centromere separation from the SPB and reductional segregation are impaired, and these processes require mating pheromone signaling (Asakawa et al. 2005; Yamamoto and Hiraoka 2003b; reviewed in Asakawa et al. 2007)

3.2

Integrity of the Telomere

Telomere specific binding proteins that ensure integrity of the telomere are required for telomere clustering in meiosis. Taz1 and Rap1 are conserved telomere binding proteins that bind telomeric DNA repeats constitutively. Taz1 directly binds to the telomere, and binding of Rap1 to the telomere is mediated by Taz1. Loss of Taz1 or Rap1 results in elongation of the telomere, but does not affect vegetative growth of the cell. However, telomere clustering in meiotic prophase is completely lost, resulting in moderate levels of reduction in recombination frequency and abnormal spore formation. This suggests that telomere clustering is crucial for the progression of meiosis (Chikashige and Hiraoka 2001; Cooper et al. 1997; Kanoh and Ishikawa 2001; Nimmo et al. 1998). In addition to Taz1, certain heterochromatin-related factors, such as Rik1 and Clr4 (but not Swi6), and Rik1 interaction proteins Dos1 and Dos2, are likewise required for proper clustering of telomeres (Tuzon et al. 2004; Li et al. 2005).

3.3

Integrity of the SPB

Telomere clustering is lost in mutants that have a defective SPB structure. Kms1 is a component of the SPB and is required only in meiosis. In the absence of Kms1, Sad1, a component of the SPB (Hagan and Yanagida 1995), disperses into several aggregates on the nuclear envelope (Shimanuki et al. 1997). In these cells, telomere clustering, homologous pairing and recombination and spore formation are impaired (Niwa et al. 2000; Shimanuki et al. 1997). Multiple aggregates of Sad1 and loss of telomere clustering are also observed in *dot* (defective organization of telomere) mutants (Jin et al. 2002). Dot2 is a homolog of human transcription factor EAP30, which regulates the level of Pcp1, a component of the SPB (Jin et al. 2005). Thus, integrity of the SPB is important in ensuring telomere clustering.

3.4

Dragging Telomeres to the SPB

Telomere clustering requires a mechanism to gather telomeres dispersed on the nuclear envelope to the SPB at the onset of meiotic prophase. Bqt1

and Bqt2, meiosis-specific proteins expressed during the mating pheromone response, are responsible for telomere clustering in *S. pombe* (Fig. 4A) (Chikashige et al. 2006; Martin-Castellanos et al. 2005; Tang et al. 2006). These proteins as a complex are able to bind with Rap1 (a telomere protein); Bqt1 is also able to bind Sad1 on the nuclear envelope (Chikashige et al. 2006). In response to the mating pheromone, Bqt1 and Bqt2 accumulate at telomeres, and Sad1 accumulates on the Bqt1/Bqt2 bound telomeres attached to the nuclear envelope. Finally, the Sad1-bound telomeres gather at the SPB to form telomere clusters (Chikashige et al. 2006). Furthermore, Sad1 interacts with Kms1 probably in the perinuclear space, and Kms1 interacts with cytoplasmic dynein (Miki et al. 2002, 2004). Thus, the Bqt1/Bqt2 complex bridges telomeres inside the nucleus to the cytoskeleton network, with Sad1 and Kms1 across the nuclear envelope (Fig. 4B) (Chikashige et al. 2006).

For tethering telomeres, two cytoplasmic dynein components, the dynein heavy chain and light chain (respectively Dhc1 and Dlc1 in *S. pombe*) likely

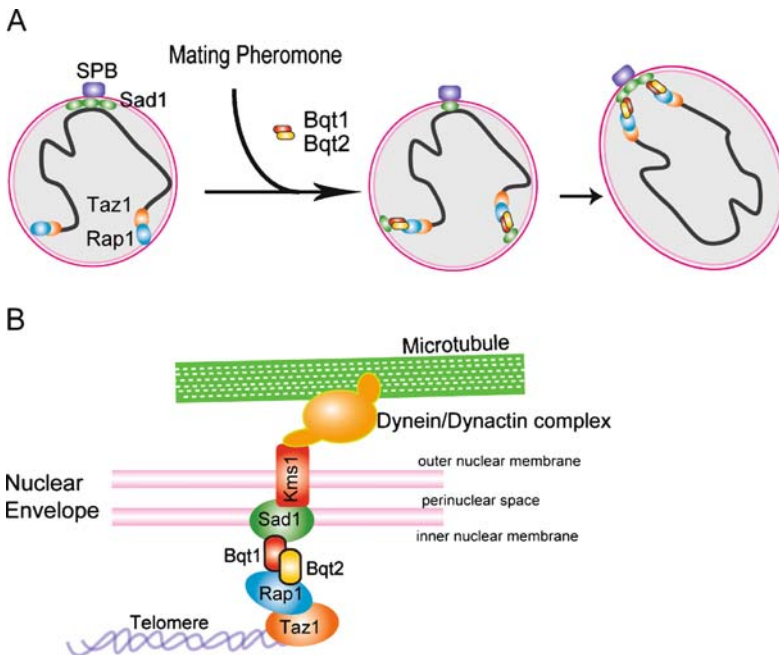


Fig. 4 Meiotic telomere-SPB complex in fission yeast. **A** During mitosis, Sad1 protein is exclusively located at the SPB. When induced by mating pheromone, Bqt1 and Bqt2 connect Sad1 to Rap1 at the telomeres on the nuclear envelope; Sad1-telomere complexes then aggregate at SPB. **B** A model for the molecular linkage that tethers the telomere to cytoplasmic microtubules. Sad1 only has one trans-membrane domain and its N-terminus interacts with Bqt1. Sad1 may interact with Kms1 in the perinuclear space and such an interaction connects the telomere to the cytoskeleton motor proteins

function. Although no obvious impairments in telomere clustering are detected in cells without dynein heavy chain Dhc1, some defects have been reported in dynein light chain Dlc1 mutants (Miki et al. 2002; Yamamoto et al. 1999). Furthermore, when both dynein heavy chain and light chain are absent, telomere clustering cannot be performed (A. Yamamoto and Y. Hiraoka, unpublished), indicating a direct role for cytoplasmic motors in telomere clustering. However, a requirement for microtubules in telomere clustering has never been directly demonstrated. In maize, bouquet formation is inhibited in the presence of colchicine, a drug which promotes microtubule depolymerization (Cowan and Cande 2002). On the other hand, bouquet formation in *S. cerevisiae* is impaired in the presence of actin inhibitors (Trelles-Sticken 2005). Involvement of microtubules and actin in both formation and maintenance of telomere clusters in *S. pombe* remains to be tested.

The finding that Sad1-Kms1 protein complex, with Bqt1/2 as a connector, moves telomeres in *S. pombe* provides a view for a general mechanism to link chromosomes to the cytoskeleton across the nuclear envelope. Sad1 and Kms1 belong to conserved SUN-domain and KASH-domain protein families, respectively. The SUN-domain proteins localize to the inner nuclear membrane, and interact with the KASH-domain proteins. KASH domain proteins are often outer nuclear membrane proteins that link the nuclear envelope with microtubules or actin filaments (reviewed in Malone et al. 2003; Tzur et al. 2006). Thus, it is proposed that SUN and KASH domain proteins are capable of moving chromosomes across the nuclear envelope with appropriate connector proteins (reviewed in Chikashige et al. 2007). Identifying the Bqt1/2 equivalents and meiosis-specific KASH domain proteins in other organisms will be of interest in the future.

4

Regulation of Nuclear Movement

Movements of the horsetail nucleus in *S. pombe* are accompanied by dramatic changes in microtubule organization, initiated by mating pheromone signaling and mediated by pulling forces generated by dynein motor complexes. Nuclear oscillation is accomplished by alternating the directions of force-generating microtubules. These processes are regulated by many coordinating factors: dynein/dynactin motor complexes, microtubules, and proteins anchoring microtubules to the cell cortex (Fig. 5).

4.1

Dynein and Dynactin

During the meiotic prophase, microtubule bundles along the long axis of the cell during the mitotic interphase (Fig. 5A) are replaced by astral arrays radi-

A



B

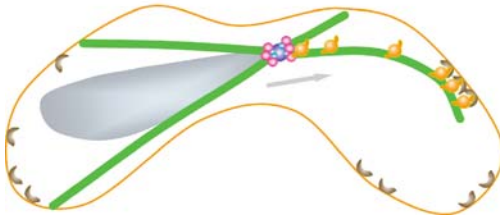


Fig. 5 Rearrangement of cytoplasmic microtubule network in meiotic prophase. **A** In mitotic interphase, microtubule bundles are parallel to the long-axis of the cell. Some microtubules associate with the SPB and are required for maintaining the central position of the nucleus. **B** In meiotic prophase, the meiosis-specific Hrs1/Mcp6 associates with the SPB and enables all microtubule bundles radiating from the SPB. The dynein/dynactin complex only exists on the microtubule bundle in the forward direction of nuclear movement. This microtubule-bundle anchors to the cell cortex where Mcp5/Num1 is localized. Mcp5/Num1 is required for accumulation of dynein at the anchor site. The asymmetrical localization of the dynein/dynactin complex may be the cause of the oscillatory nuclear movement in meiotic prophase

ating from the SPB (Ding et al. 1998; Svoboda et al. 1995) (Fig. 5B). Dynamic polymerization and depolymerization of astral microtubules directly contribute to nuclear movements. Disruption of microtubules by thiabendazole eliminates nuclear movements (Ding et al. 1998). Cytoplasmic dynein components, Dhc1 and Dlc1, are required for oscillatory nuclear movement. Dhc1 and Dlc1 are present along the microtubule in the forward direction of movement (forward-extending microtubules) as well as at microtubule-anchoring sites of the cell cortex. This asymmetrical localization generates the pulling force to move the nucleus toward the cell cortex, and alternating the direction of force-generating microtubules leads to the oscillatory movement (Miki et al. 2002; Yamamoto et al. 1999, 2001). Dynein also localizes to the SPB, and dynein light chain Dlc1 interacts with Sad1 (as shown in two-hybrid analysis, Miki et al. 2002). Double mutation of Dhc1 and Dlc1 causes more severe defects in karyogamy, recombination and spore formation than either single mutation, suggesting that the functions of Dhc1 and Dlc1 may be redundant but are not completely overlapped (Miki et al. 2002).

Dynactin is a dynein-associated protein complex required for most dynein-mediated activities. The dynactin complex includes Glued, a coiled-coil protein that interacts with microtubules and cytoplasmic dynein. Ssm4, a meiosis-specific Glued subunit in *S. pombe*, is required for oscillatory nuclear movement (Niccoli et al. 2004; Yamashita et al. 1997). Ssm4 associates with Dhc1 and together with the CLIP-170 homolog Tip1 regulates Dhc1 localization (Niccoli et al. 2004). In addition to dynactin, Num1/ Mcp5, a conserved PH-domain protein that functions in nuclear migration, is also required for anchoring dynein at the cell cortex and is therefore necessary for the oscillatory movement (Saito et al. 2006; Yamashita and Yamamoto 2006). The dynein/dynactin-mediated nuclear migration is also associated with many biological processes such as nuclear migration or positioning during development and morphogenesis in general (Yamamoto and Hiraoka 2003a).

4.2

Concentrating the Microtubule Bundles at the SPB

Reconstruction of the microtubule network upon mating pheromone induction is controlled by a meiosis-specific coiled-coil protein Hrs1/Mcp6 (Saito et al. 2005; Tanaka et al. 2005). In the absence of Hrs1/Mcp6, the microtubule bundles radiating from the SPB are not stable. These bundles disperse from the SPB and form a mitotic interphase-like parallel microtubule network, and as a result, nuclear oscillation is compromised (Saito et al. 2005; Tanaka et al. 2005). Hrs1 localizes at SPB and interacts with γ -tubulin complex components Alp4, Mto1 and the N-terminal portion of Kms1. Thus, it may play a role in attracting the minus-end of microtubules via the γ -tubulin complex and tethering them to the SPB (Tanaka et al. 2005). An additional factor, Ase1, a microtubule binding protein required for bundling microtubules in the spindle, is also required for formation of the microtubule bundle in meiotic prophase and for nuclear oscillatory movement (Yamashita et al. 2005).

5

Conclusion and Outlook

Here we have summarized recent progress in the analysis of meiotic prophase events related to the pre-alignment of homologous chromosomes in an asynaptic organism, *S. pombe*. The requirement for telomere clustering, nuclear movement and recombination in the alignment and pairing between homologous chromosomes has been clearly shown in this organism. However, as in other organisms, the mechanism by which pre-aligned homologous chromosomes recognize each other is unknown (Gerton and Hawley 2005). In *S. pombe*, centromere pairing is independent of recombination (Ding et al.

2004). Thus, centromeres could be potential recognition sites in homologous chromosome pairing.

In a canonical synaptic organism, the budding yeast *S. cerevisiae*, the telomere clustering occurs transiently in zygotene. Ndj1, a meiosis specific telomere binding protein which tethers telomeres to nuclear envelope, plays a central role in telomere clustering in this organism; absence of Ndj1 causes defects in telomere clustering and in several steps of homologous recombination, as well as some delay in pairing and synapsis (Chua et al. 1997; Conrad et al. 1997; Trelles-Sticken et al. 2000; Wu and Burgess 2006). How telomere clustering effects the core recombination steps in the context beyond homologous chromosome alignment in both synaptic and asynaptic organisms remains to be explored.

The mechanisms involved in homologous pairing are diverse among organisms. Retaining telomeres in a tight cluster and dramatic oscillatory nuclear movements may be unique for *S. pombe*. However, as bouquet formation during meiotic prophase is conserved in most organisms, as is pre-synaptic alignment of homologous chromosomes, dissection of the molecular machinery for these processes in *S. pombe* can provide valuable insights for analysis in synaptic organisms as well. Furthermore, the conserved attachment of telomeres to the nuclear membrane supposedly reflects a very ancient means of linking eukaryotic chromosomes to cytoskeletal structure, which may have assisted proto-mitotic microtubular mechanisms in segregating the genome. Its predominant survival in meiotic prophase is in line with assuming an evolutionarily ancient origin of meiosis (see Egel and Penny, 2007, in this volume). Its upgrading to the primary means of meiotic homolog alignment, however, appears to be a relatively recent event peculiar to the lineage of fission yeast.

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On the Origin of Meiosis in Eukaryotic Evolution: Coevolution of Meiosis and Mitosis from Feeble Beginnings

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Abstract The processes making up meiosis are complex and, arguably, may be the most complicated cellular process in eukaryotes. Its origin has been discussed at length for over 100 years, without any consensus. Although earlier investigators favored it as being very ancient, there has been a tendency over recent decades to consider it as a feature that arose relatively late in the evolution of eukaryotes. However, the study of the genomes of early-diverging eukaryotes makes it appear that many proteins that are specific and/or required for meiosis occur widely throughout eukaryotes, implying that meiosis must have evolved at least before their last common ancestor. Indeed, the advantages of genetic recombination would have applied to much earlier forms of life, right back to the hypothesized RNA world. In agreement with a very early origin, many of the proteins involved in meiosis, especially those involved in recombination and/or in repair of double-stranded DNA breaks (DSBs), have homologs in prokaryotes. The simplest hypothesis at present is that some form of recombination would have occurred extremely early in life (possibly even before the origin of protein synthesis) and it is likely that there was always some form of genetic recombination since that time. At some point in early evolution, a more specialized regimen of abundant recombination was uncoupled from the repair of accidental damage to serve a once-per-lifecycle event of genomic reconstitution. This happened before the last common eukaryotic ancestor, when the mitotic system in the modern sense had not yet been fully consolidated. We call this the Coevolution Hypothesis for the origin of meiosis—that both mitotic segregation mechanisms and meiotic recombination and chromosome reshuffling occurred very early and kept improving with increasing complexity during the evolution of eukaryotes.

Abbreviations

DSB	double-strand break
ESP	eukaryotic signature protein
G-protein	GTP-binding protein
LTG	lateral gene transfer
LUCA	last universal common ancestor
MMR	mismatch repair
RNAi	interfering RNA
PSF	periodically selected function
snRNA	small nuclear RNA

1

Introduction

The impact of sexual reproduction on the evolution of eukaryotes can hardly go unnoticed. It strongly influences the physical appearance of most multicellular organisms, including the prominent reproductive organs of flowering plants and the sexually dimorphic body parts and behavioral patterns of animals. Nevertheless, the early evolution of meiosis is still considered puzzling to many authors and a consensus on its origin has not yet been reached. Most other chapters of this book (or the accompanying volume) focus on the mechanism of meiotic recombination in experimental model organisms. Here we consider how the meiotic system may have originated at the dawn of eukaryotic evolution, and the likely driving forces that have favored this important trait. As these questions relate to historical events that cannot yet be reconstructed, potential answers can only be proposed as plausible hypotheses, based on the genes that affect some aspect of meiosis and that are widely distributed in eukaryotes. In our quest for the likely origin of meiosis at the level of protoeukaryotes we will strictly confine our considerations to unicellular organisms. The related issues of the maintenance of meiotic sex in multicellular eukaryotes are dealt with in accompanying chapters (D.-H. Lankenau, this BOOK; I. Schön, D.K. Lamatsch and K. Martens, this BOOK).

A real problem is the sheer complexity of meiosis. The full cycle involves cell fusion (syngamy), nuclear fusion (karyogamy), chromosome recognition, pairing and synapsis, controlled cutting of the DNA (DSBs), crossing over and recombination, chromosome doubling and then two cell divisions with reduction to haploid cells (see Neale and Keeney 2006; or the introductory chapter of R. Egel, this SERIES). The apparent complexity led a well-known early cytologist (Darlington 1958, p 214) to despair that meiosis could have arisen by normal micro-evolutionary processes (that is, by Darwinian evolution). He could not imagine all the component steps being useful in their own right, before being co-opted into meiosis. Part of our purpose here is to update a previous analysis (Penny 1985) showing that—even though we do not know the detailed historical pathways—meiosis can be understood in a standard evolutionary manner as an incremental series of stages, each of which is functional. The availability of large numbers of genomes and the identification of protein function by comparison is improving our ability to reconstruct hypotheses about the evolution of meiosis that, in principle, lead to testable predictions. The process is helped because we are increasingly getting a better picture of the cell of the last eukaryote common ancestor (Kurland et al. 2006; Poole and Penny 2007a,b).

The main hypothesis put forward in this essay is developed along the following steps:

- The “invention” of meiosis dates back to before the last common ancestor of extant eukaryotes, since a core set of meiotic functions is present in essentially all major branches of eukaryotes. How much earlier this has happened can hardly be inferred from sequence comparisons alone.
- Meiosis thus joins with other complex systems that are common to essentially all eukaryotes, such as mitosis, basic chromosome organization, nuclear envelope and pore complexes, cytoplasmic membrane trafficking, exo- and endocytosis across the cytoplasmic membrane, cytoskeleton and motility, microtubules and 9+2 flagellar structure, etc. Hence, a plausible theory on the origin of meiosis very much depends on the more general conception how the complex eukaryotic cell machinery has developed—in contrast to both archaeal and bacterial organization.
- How do the three principal branches of Eukaryota, Archaea, and Eubacteria most likely relate to their “last universal common ancestor” (LUCA)? Any reasonable answer to this evolutionary enigma has immediate bearing on the presumptive boundary conditions for the origin and early evolution of meiosis.
- According to Jeffares et al. (1998), the survival in eukaryotes of several “molecular fossils” from the putative RNA world suggests a direct link of continuous evolution, rather than a detour via “prokaryotic” intermediates¹.
- The numerical kinetics of evolution must have been radically different under primordial conditions (during the RNA age and shortly thereafter) as compared to the modern biosphere. This can be deduced from elementary assumptions. A theory applying to relatively simple, single molecules of RNA/DNA has been developed by Manfred Eigen several decades ago as the quasi-species concept, but the implications of this framework for more complex protocells has not yet been widely appreciated.
- We try to combine this concept with Carl Woese’s suggestion that the age of LUCA signified a critical turning point or “phase transition” between different modes of evolutionary change in relation to ever decreasing error rates of replication. As a corollary to the direct link to complex protoeukaryotes, we argue for a selective genome reduction in the establishment of “prokaryotic” descendants away from the main stem lineage leading to eukaryotes.
- A number of early features assumed for protoeukaryotes can, in fact, be considered “preadaptive” for early meiosis, which renders the emergence of the meiotic system less difficult to understand than its *de novo* “invention” at a later stage.
- It is then argued that the meiotic program of crossover-coupled genome reduction primarily evolved to protect a sizeable part of the genome from deteriorating by cryptic mutations, which are not purged by immedi-

¹ In this paper, we use *prokaryotes* and *prokaryotic* to indicate the overall level of organization represented by extant bacteria and archaea, rather than a monophyletic clade of organisms (see Sapp 2006).

ate selection. In particular, this applies to the extensive set of essential genes that are only used at the rare occasion of preparing for a state of dormancy—by encystation or spore formation—allowing survival in severe environmental crises that recur periodically.

- As a summing-up of the preceding points, we try to formulate a Coevolution Hypothesis for the origin of both meiosis and mitosis from very early in the protoeukaryotic lineage.

2

A Conserved Core of Meiotic Proteins

The universal tree, comprising Bacteria, Archaea, and Eukarya (Woese et al. 1990; Brown and Doolittle 1997), is based on the comparison of genomic sequences on a large scale, which supports the phylogenetic status of archaea and eukaryotes as sister lineages—to the early exclusion of the bacterial side branch. The clustered affinity is particularly pronounced for translational activities relating to the ribosome, as well as replication and other DNA-related functions. The origin of meiosis, too, could be placed on this timeline by the comparison of appropriate sequences. As mentioned further below, research on yeast genomes has pioneered the characterization of meiotic proteomes, describing the set of genes preferentially expressed during meiosis (Mata et al. 2002; Chu et al. 1998). Whilst many of these are specific to one of the yeasts, or only to fungi, others have orthologs in other phyla. Fewer still have orthologs in all the phyla looked at, and these comprise the most interesting set from a phylogenetic perspective.

Villeneuve and Hillers (2001) defined a “core meiotic recombination machinery” from such a comparison, and Ramesh et al. (2005) demonstrated that the early protist lineage *Giardia* had this core set. These proteins fall into two subsets of overlapping functions—a more general set that is also involved in important functions in mitotic cells, and a specialized set to be used in meiosis exclusively. The number of these conserved meiotic proteins is likely to increase in the future, because more genomes are being sequenced and more structural folding information is available for homology grouping (where sequences are otherwise too diverged to be aligned). The molecular functions of these proteins are extensively reviewed in other chapters of this book or series, so they are very briefly summarized as follows, referring to gene symbols as used in the yeast *Saccharomyces cerevisiae* (underlined, for the meiosis-specific subset).

- The homologous pairing partners are aligned in register by synaptic filaments (Hop1, Hop2, Mnd1).
- Double-strand breaks are introduced deliberately by Spo11, related to archaeal topoisomerase VI.
- The 5' ends are resected by nucleases (Rad50, Mre11).

- (iv) 3' ssDNA is mobilized by Rad52 for homology search and heteroduplex formation by helical Rad51 and Dmc1 filaments.
- (v) Heteroduplex DNA is processed by mismatch repair-related functions (Mlh1, Mlh2, Mlh3, Pms1; Msh2, Msh6, Msh4, Msh5).

Prime candidates for a likely extension of this list could be meiosis-specific cohesin (Rec8) for differential sister chromatid coherence, and shugoshin (Sgo1) for sister centromere connectivity in meiosis I; but the conservation of these functions beyond the fungal/animal lineage has not yet been fully explored. Thus, DNA damage repair proteins, in particular, have been re-recruited and modified for the meiotic program (Marcon and Moens 2005). The proteins are well characterized in yeasts, but multicellular eukaryotes, such as *Drosophila* or *Arabidopsis*, are now also good experimental systems for understanding the functions of these genes (see D.-H. Lankenau 2006; and various topical chapters in this BOOK or SERIES).

The widespread occurrence of meiosis-specific proteins throughout the major eukaryotic lineages suggests that the meiotic system was present in the common eukaryotic ancestor. As to the prehistory of the protoeukaryotic lineage, however, there is a lot of speculation but rather little critical evidence. In the following we shall pick and choose among such speculations so as to find the least cumbersome way of explaining the putative origin of meiosis.

3

The Complex Eukaryotic Signature

It is a truism that eukaryotes are more complex than prokaryotes (Bacteria, as well as Archaea); how eukaryotes got there is yet a different matter. For one thing, modern eukaryotes have a nucleus and organelles, whereas prokaryotes do not. Furthermore, the cooperation of interactive protein machines in eukaryotes is much more sophisticated than in their prokaryotic counterparts. Among the host of hypotheses to explain the origin of eukaryotic cells, the only undisputed theories concern the endosymbiotic origin of mitochondria and chloroplasts². The internalization of protomitochondria, in particular, likely preceded the latest common ancestor of all extant eukaryotes (Embley and Martin 2006). As to nature and prehistory of the protoeukaryotic host of mitochondrial symbiosis, however, there is much less consensus³.

² Both mitochondria and chloroplasts still carry rudimentary genomes, which relate these organelles to α -proteobacteria and cyanobacteria, respectively.

³ Among the various archaeal/bacterial fusion hypotheses for the premitochondrial protoeukaryotic host lineage, the "hydrogen hypothesis" (Martin and Müller 1998) is perhaps the soundest proposal in physiological terms. From genomic comparisons, however, there is no clearcut evidence for such a fusion. Seen in the Woesean perspective (Sect. 7), up to the era around the last common ancestor, there may actually have existed many physiologically differentiated sub-lineages that more or less freely merged their cytoplasm and/or traded their genes, but their "genealogical identity" decayed so rapidly with time that we will never know about their existence from sequence alignments of extant genomes.

Notably, a set of ~350 “eukaryotic signature proteins” (ESPs) has been singled out—with a broadly conserved representation throughout eukaryotes, but little to no significant homology to proteins in Archaea or Bacteria (Hartman and Fedorov 2002). These proteins are associated with protein synthesis or turnover (cytoplasmic ribosomes, proteasome, etc.), cytoskeleton (actin, tubulins, etc.), membrane trafficking (lipid anchors, exo-/endo-cytosis, etc.), signalling systems (calmodulin, ubiquitin, G-proteins, cyclin-dependent kinases, etc.), and the nucleus (histones, RNA polymerase subunits, nucleolar proteins, spliceosomes, nuclear pore complexes, etc.)—even an RNA-directed RNA polymerase involved in the RNAi reaction⁴, without homology to viral proteins.

It is indeed remarkable how many putative relics there are in eukaryotes, which may date back to the RNA world (Reaney 1974, 1984; Forterre 1995; Poole et al. 1998; Penny and Poole 1999; Kurland et al. 2006; Rodriguez-Trelles et al. 2006). In eukaryotes, we observe widespread RNA involvement in the synthesis of DNA and RNA; the processing of RNA; transport of RNA within the nucleus and between the nucleus and cytoplasm; and in the regulation of RNA (such as RNAi and riboswitches). For example, it appears that the spliceosome (with its five snRNAs⁵ and over a hundred proteins) was present in the last common ancestor of eukaryotes (Collins and Penny 2005). Indeed, the minor spliceosome⁶ also appears ancient in eukaryotes (Russell et al. 2006). Whatever interpretation is adopted, it is interesting to note that so much of the cellular infrastructure involving RNA takes place in the nucleus—or involves transport of RNA in and out of the nucleus. These RNA processes are, in addition to a presumptive early syncytial stage of life (see below) with widespread membrane sharing, are still observed only in eukaryotes. The nuclear envelope itself, together with its gating pore complexes, may be an ancient relic from such a syncytial era.

Noteworthy, the ends of linear eukaryotic chromosomes are commonly being maintained by telomerase, which is a reverse transcriptase with a built-in RNA template for DNA synthesis at telomeric repeats (Chan and Blackburn 2004). This specialized ribonucleoprotein is considered one of the most conspicuous relics of the RNA world in eukaryotes (Meli et al. 2001; see Sect. 5)—likewise indicative of an ancient root. Somewhat surprisingly, telomerase was not included in this common set of ESPs. Telomerase-dependent telomeres are indeed missing in certain eukaryotes⁷, but somewhat divergent, putative

⁴ Small “interfering” RNAs are processed from dsRNA, part of which is synthesized by a specialized RNA-directed RNA polymerase (Baulcombe 2006).

⁵ snRNAs: small nuclear RNAs, mostly involved with splicing introns, always associated with specific proteins.

⁶ Structurally similar to common spliceosomes, the diverged “minor spliceosomes” remove a group of atypical introns (U12-type), which are present in animals and plants, but not in fungi.

⁷ For instance, the terminal sequences of *Drosophila* chromosomes are derived from transposons (Levis et al. 1993).

telomerase genes have been detected in *Giardia* and *Caenorhabditis* (Malik et al. 2000).

Certain ESPs (such as actin, tubulins, histones, and ubiquitin) have shown extraordinary constancy throughout diverse eukaryote lineages. This is commonly ascribed to multiple interactions in structurally constrained, oligomeric complexes, which allow little change of amino acid sequence. When these functionally important protein machines had been perfected early on in protoeukaryotic evolution, the rate of change was dramatically reduced thereafter. Presumably, such multiple perfection has taken considerable time, which suggests that the eukaryotic lineage is much older than some other proteins may indicate (Doolittle 1995).

It has long been maintained that the eukaryote ancestor that tamed the mitochondrion was capable of phagocytosis (see Cavalier-Smith 2002); moreover, both amoeboid and flagellate motility are deeply engrained in the common eukaryotic heritage. From the diversification of G-proteins as signalling components it appears that the most ancient branches where involved in secretion, before others became engaged in phagocytosis (Jékely 2003). Also, G-protein-coupled receptors are deeply involved in nutritional signalling (see Hoffman 2005; Prabhu and Eichinger 2006), in addition to their employment in the recognition of potential mating partners and other cell-cell interactions.

4

The Universal Trifurcation

In the Woesean trichotomy of cellular life forms, there are actually four nodal branches to be related to one another: in addition to the extant domains, or superkingdoms, of Bacteria, Archaea, and Eukarya, the fourth branch of interest is the putative primordial lineage leading up to the last universal common ancestor (LUCA), on which we have no direct observations. For the numerous household genes that are represented in all three superkingdoms, it is safe to assume that they were present in LUCA as well. In all other cases, however, we are on much shakier ground. Individual genes can have been gained or lost anywhere in any lineage, and they can have shuttled between domains by lateral gene transfer (LTG)⁸. Moreover, reasonable guesses at the organizational status or life style of the LUCA are yet harder to substantiate.

The universal tree was originally disclosed by large-scale comparison of rDNA sequences (Woese et al. 1990), which since have been supplemented by sequences of other genes. This tree makes the monophyletic eukaryote lineage the sister group to archaea—to the exclusion of the more divergent bacteria. Somewhat surprisingly, rather few other genes reveal the same phylogenetic

⁸ also referred to as horizontal gene transfer (HGT).

pattern as the pioneering rRNAs, and most of those encode a common core of ribosomal proteins, DNA/RNA polymerase subunits or elongation factors (Harris et al. 2003). To account for the disturbing influence of LTG on phylogenetic analyses, the method of “conditioned reconstruction” has been proposed and applied to the common ancestor problem, deriving at the novel concept of the “ring of life” (Lake and Rivera 2004), where eukaryotes are conceived as mergers of two or more prokaryotic genomes. Yet, the validity of this approach has been contested (Baptiste and Walsh 2005), so its conclusiveness remains to be resolved.

Traditionally, proposals for LUCA’s general appearance were modelled mostly in resemblance with a prokaryotic cell, setting eukaryotes aside as derivative latecomers (see Simonson et al. 2005). This is not, however, the only conceivable scenario (see Poole et al. 1998, 1999). At the supra-molecular level, there are two primordial traits in particular that may have been passed on to protoeukaryotes directly from the pre-LUCA lineage⁹.

- (i) Somewhere in the pre-LUCA era, the evolution of nucleic acid replicators must have started from gene-sized pieces, most likely linear molecules. This makes the congregation of the entire genome on a circular chromosome a derived feature, which presently is found in both bacteria and archaea, but not in eukaryotes. – Has this significant property been given up again in protoeukaryotes or has it been established twice in prokaryotes, perhaps transferred from one superkingdom to the other by LTG? – The latter possibility cannot be rejected by statistical arguments alone, and we are inclined to the view that multiple linear chromosomes (with telomeres) have been inherited by protoeukaryotes from the pre-LUCA lineage.
- (ii) The earliest cell-like assemblies of living matter were probably not yet surrounded by rigid boundaries. Hence, the existence of cell walls is a derived feature, which presently is found in most bacteria and archaea¹⁰, but not in most protist eukaryotes. We are inclined to presume that there had never existed cell walls in the protoeukaryotic lineage.

Until now, by extrapolation from the extant superkingdoms, we have sketched the presentation of LUCA as if this hypothetical hub of evolution was equivalent to other nodes in the subsequent branching pattern of the universal tree of life. As it will become apparent later (Sects. 6 and 7), this is an inappropriate assumption. It can be argued that the stage of LUCA represents a critical phase transition between two modes of evolution (Woese 1998). Furthermore, it is not self-evident that the common ancestor should have carried a fully consolidated genome based on double-stranded DNA, even though all three extant

⁹ Additional traits linking eukaryotes to the pre-LUCA era at the RNA level will be pointed out below (Sect. 5).

¹⁰ *Mycoplasma*-like bacteria and *Thermoplasma*-like archaea do not have rigid cell walls either. Certain thermophilic acidophilic sulfur-metabolizing archaea without rigid cell walls have been discussed as a potential “preadaptation” for the evolution of eukaryotic cells (Searcy and Hixon 1991).

superkingdoms are organized this way. This reservation is based on the disturbing absence of homology of the replicative DNA polymerases in bacteria and archaea/eukaryotes (Leipe et al. 1999). Conceivably, DNA-based replication was first developed by various viruses and/or plasmids that propagated in the originally RNA-based cellular entities (Forterre 2002, 2006; Koonin 2006).

Our aim in this study is to be as independent as possible of the real (but unknown) position of the root of the eukaryote tree. We currently use the tree of Keeling et al. (2005) as a research focus but that tree (considering it as unrooted) can be resolved in a large number of ways ($\sim 10^6$). However, if we find a gene in all the main lineages of standard eukaryotes (those without highly reduced and/or specialized genomes), then we can infer the gene occurred in the last common ancestor of extant eukaryotes. Examples of such reasoning include the finding that all major lineages of eukaryotes have a major spliceosome (Collins and Penny 2005) and that even groups considered to be primitive, such as *Giardia* where meiosis has never been observed, do have most of the necessary enzymes (Ramesh et al. 2005). Perhaps, the appropriate conditions for *Giardia* to undergo meiosis have not yet been discovered (Birky 2005). Similar findings extend to *Entamoeba* (Stanley 2005).

5

The RNA World Scenario

To better understand the prehistory of the primordial lineage before the last common ancestor, it is necessary to briefly outline the RNA world hypothesis; this is currently the main hypothesis for the succession of stages during the later stages of the origin of life. The RNA world hypothesis simply states that,

- RNA preceded (encoded) proteins as macromolecular catalysts, and
- RNA preceded DNA as a coding and replicating macromolecule¹¹.

The literature is extensive but is summarized in Penny (2005; cf. Forterre and Gribaldo 2007). The evidence comes from the diverse roles of RNA in modern cells, including information storage (mRNA), translation (tRNA), protein synthesis (rRNA), DNA synthesis (initial RNA strands), ribonucleotides being precursors for deoxyribonucleotides, the existence of catalytic RNA (ribozymes), rRNA modification (mediated by snRNAs)¹², riboswitches¹³, and

¹¹ The least understood aspect of the RNA world scenario is still the prebiotic formation and oligomerization of the first ribonucleotides, which are rather unstable under most geochemical conditions. Interestingly, at very low temperatures (in sea ice, for example) RNA molecules up to several hundred nucleotides can form (Trinks et al. 2005), though we do not know yet whether replication occurs at this temperature.

¹² snRNAs: small nuclear RNAs (noncoding).

¹³ Riboswitches are metabolite-binding RNA domains, mostly in non-coding regions of mRNA, serving regulatory roles in both prokaryotes and eukaryotes (Epshtein et al. 2003; Sudarsan et al. 2003).

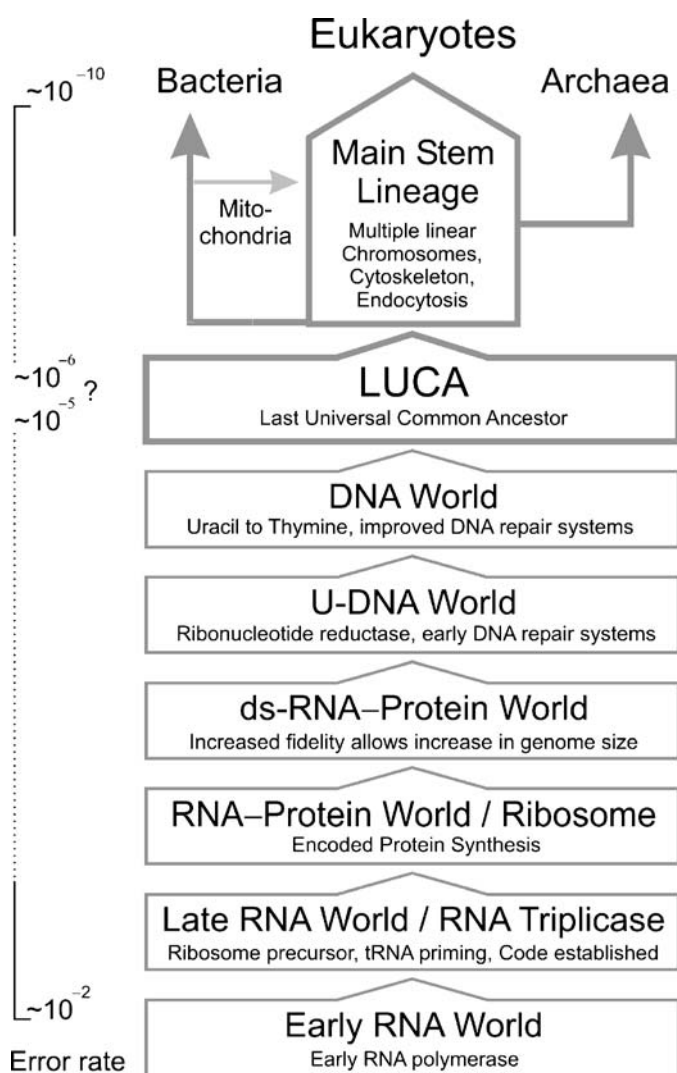
Fig. 1 Overview of steps from the prebiotic world to eukaryotes and prokaryotes, emphasizing a continued increase in replication accuracy. A “timeline” of events can be deduced, with the RNA world currently being the earliest identifiable form of “genetic life”. Translation by the “all-RNA” prototype ribosome occurred to produce the first proteins—low complexity RNA chaperones that we expect would have increased replication accuracy—allowing a larger genome size. By the time of LUCA (last universal common ancestor), both catalytic proteins and DNA had arisen with improved accuracy—still considerably lower than that of present life. The major stem lineage then developed into eukaryotes directly, whilst the two prokaryotic side lines split off under regressive selection for minimum size and a single circular chromosome (based on Poole et al. 1999; Woese 1998). Presumably, the major stem lineage with many linear minichromosomes did not increase replication accuracy as rapidly as the two “prokaryotic” sidelines. Endosymbiosis by bacterial protomitochondria completed the basic organization of eukaryotic cells. (U-DNA: possible intermediate containing uracil instead of thymine) ▶

the regulation of RNA expression (RNAi). Artificial selection experiments to probe the potential of ribozyme activities *in vitro* have indeed come a long way in a surprisingly short time (e.g., Bartel and Unrau 1999; Lawrence and Bartel 2003). It is also usually assumed that (encoded) proteins preceded DNA, and this gives the sequence RNA world → RNP world → DNA world (as in the present), where RNP is a ribonucleotide-protein world before the evolution of DNA (see Poole et al. 2001). It is likely that there were earlier proto-living systems, but for the present purposes, we need only consider RNA, protein and DNA because it is the accuracy (fidelity) of replication that is an important issue for the origin of recombination and meiosis. Of particular interest are the spliceosomal ribozymes that catalyse the intra- or intermolecular joining of particular RNA sequences (respectively in *cis* or in *trans*), which links the occurrence of genetic recombination to the primordial RNA world scenario¹⁴

From our knowledge of the lower accuracy and slower speed of ribozymes (compared to protein catalysts, see Jeffares et al. 1998) it is expected that replication fidelity would have been much lower in an RNA world. Figure 1 represents this as increases in replication accuracy from about one error in 10^2 nt to around 10^{10} nt (or better) in the modern DNA world (Drake 1991)¹⁵. This drawing extends on previous schemes of Poole et al. (1999) and Penny (2005). The conclusions we draw here relate to a continued increase in replication fidelity, the continued existence of recombination, and the evolution of the control over DSBs in DNA. From theoretical considerations alone (see Sect. 6; Eigen 1992), it is inevitable that the increases in maximum size of

¹⁴ Of the vivid and still-ongoing debate over “introns early” vs. “introns late” in evolution, one of the most ingenious notions suggests an ancient function for introns as discriminatory signals between coding and non-coding RNA strands. Only the spliced coding strands would act as messengers for protein synthesis, whereas both non-spliced strands would serve as propagative molecules for replication and heritability (Fedorov and Fedorova 2004; Hastings 2005).

¹⁵ The starting value is set around one error in 10^2 nt, since functional RNA sequences about 100 nt long appear sensible to begin with.



individual chromosomes during evolution from the RNA- to RNP- to DNA worlds must have depended on corresponding increases in replication fidelity. Thus we expect any early RNA system to be relatively small for any particular unit of replication, though several independent units ("chromosomes") are likely. Under such circumstances, recombination would be particularly advantageous (Bernstein et al. 1984).

Yet, as we cannot know how fast the coevolution of increasing chromosome size and decreasing error rate has occurred relative to the various intermediate stages, not even an approximate scaling has been given at the left-hand side of Fig. 1; the important point is the accuracy would keep increasing. For sub-

sequent arguments it is relevant to assume that the error rate at the time of LUCA was still close to the corresponding Eigen threshold (Sect. 6), but the absolute value of the overall threshold would depend on the size of the chromosome(s), the number of which we cannot determine. As mentioned above, we do not share the predominant view held by others that the LUCA carried a single chromosome of prokaryotic organization. From all that we know of the genetic advantages of recombination there would be a strong selective advantage of recombination in the earliest RNA replicating systems, and indeed RNA viruses show recombination (Worobey and Holmes 1999). The advantages of recombination are not limited in any way to modern (DNA-based) organisms.

Lehman (2003) outlines four reasons why recombination may have arisen in the proposed RNA world. The first is simply that recombination is found in all groups (archaea, bacteria, and eukaryotes) as well as in viruses. Thus, it is genuinely universal in a phylogenetic sense. Secondly, recombination is considered important in the modern world as an escape from "Muller's ratchet" (the continued build-up of slightly deleterious mutations in the genome, see D.-H. Lankenau, this BOOK). Lehman points out that the problem would be even more acute in an earlier RNA world where the error rate of replication would be considerably higher than in modern DNA-based organisms. Thirdly, splicing systems are possible analogs of recombination, and trans-splicing (between separate RNA molecules) has been observed in several eukaryotes. Indeed, Lehman sees splicing as a relatively easy way for increasing the length of RNA transcripts, without the use of high-energy intermediates. A similar mechanism may have added triplets of nucleotides as possible precursor for the origin of protein synthesis on the ribosome (Penny 2005).

Finally, Lehman argues that in the absence of high-accuracy replication, the only way to build up long genomes would be to synthesize shorter pieces and then "combine" them. A possible analogy is the modern influenza virus (see Fujii et al. 2003) where the genome is in eight fragments (each within the Eigen limit of genome size for RNA-based replication), although in this case the assembly into the virus does not require the fragments to be physically linked into one long molecule. Lehman's approach is speculative, but it is perhaps the only hypothesis we have at present that allows the build-up of large genomes in the presence of a relatively high error rate during replication. The main point though is that recombination would be even more advantageous in an RNA world than it is today. The same argument of a fragmented genome, with many linear chromosomes staying below their own respective Eigen limits, should prevail throughout the main stem lineage leading to eukaryotes, as suggested in Fig. 1.

Similarly, we expect that proteins for the control and/or repair of DSBs in DNA would have arisen simultaneously with the invention of DNA, and its takeover of the main coding role for the cell. Thus we expect that both some form of recombination, and the control of DSB repair, would have occurred continuously from before the division into archaea, bacteria, and eukaryotes.

This is not to say that all the current systems are homologous (new proteins could have evolved or been co-opted from related processes), just that all intermediate organisms would have had some form of these two processes—without the capability of DSB repair, a lineage is effectively terminated.

6

Dynamic Implications of Eigen's Quasi-Species Concept

The quasi-species concept of Eigen and Schuster (1977) showed that the accuracy of replication placed limits on the size of genome¹⁶ that can be maintained by selection (see also Eigen 1992; Maynard Smith and Szathmary 1995, p 44). The higher the error rate during replication, the smaller the maximum permissible genome size—the critical “Eigen limit”¹⁷; if the genome is too large for the mutation rate, the whole system degrades in an “error cascade” or “mutational meltdown” and cannot be inherited in a recognizable lineage. On the other hand, the speed of evolutionary change—or “evolvability”—is highest just below the Eigen limit.

Eigen's quasi-species concept has indeed revolutionized the mathematical foundations for Darwinian evolution in general, and RNA viral evolution in particular. Instead of considering multiple mutations individually and responding to a given selection pressure, the quasi-species model follows distributions of related sequences through the uncountable paths of multi-dimensional (yet finite) sequence space. When such dynamic distributions are subject to selection, long-term survival depends both on the fitness of individual sequences and the distribution of fitness of related sequences. This gives unprecedented relevance to the many individually neutral mutations that can bridge considerable depressions in the fitness-scape between different peaks of higher suitability (see Eigen 1992, p 98). In other words, if there are many sharp peaks on the fitness landscape, then part of the evolving system will almost always be trapped on a local optimum. But if some of these peaks are more like flat and massive mesas, these may well be able to dominate the quasi-species distribution of related sequences by the number of accumulating individuals. Also, more ridge-like connections may extend from such broader mesas to neighboring tops of a higher absolute fitness than from narrow, isolated peaks. As a twist on Darwin's well-known theorem, such modified population effects have been popularized as “the survival of the flattest” (Wilke et al. 2001; Wilke 2005).

¹⁶ In this context, as originally developed for RNA viruses, “genome” stands for a single molecule carrying all the genes. In a composite genome, the length of individual chromosomes is limited by the Eigen threshold according to the momentary error rate. The mathematics of how the overall threshold might deviate if the genome consisted of a large number of rather small chromosomes has not yet been critically explored.

¹⁷ Roughly speaking, the Eigen limit allows about one error (on average) per maximum genome length per round of replication.

In the present context, the most relevant aspects of the quasi-species concept are its dynamic implications. At high mutation rates, even the best-fit individuals are not represented in future generations by identical sequences, but will inevitably be surrounded by mutated, related sequences. The most generally appreciated feature comprises the characteristic Eigen limit—a threshold function that relates any given length of sequence to its highest allowable mutation rate; beyond this limit the sequence is degraded in an informational melt-down crisis (see Eigen 1992, p 83)—there are just too many errors for the sequence length to preserve its informational content. As a corollary to this, yet little referred to elsewhere, the innovative speed of evolution is highest just below the critical Eigen limit (*ibid.* p 84). Compared to real biological entities, the axiomatic quasi-species model is idealized by focusing on the multiple paths of nucleotide mutations in sequence elements, without accounting for the additional effect from deletions and/or insertions of sequences, nor the shuffling of multiple chromosomes and/or the recombinational rearrangement between equivalent (homologous) chromosomes. The systematic inclusion of chromosome assortment and crossovers, of course, is the hallmark of meiotic sex in eukaryotes, the gene pools of which develop as more or less panmictic populations. Yet, the more sporadic exchange by parasexual means at the prokaryotic level can likewise affect the genome distribution of entire populations.

Returning to the developmental scheme of Fig. 1, the decreasing rate of replication errors is not the only trend. Simultaneously, the large number of gene-sized pieces at the beginning must have developed further. Whilst the total number of functional genes could have increased, the number of different sequence entities has given way to fewer classes of concatenates, culminating in circular chromosomes that carry the entire genomes of bacterial or archaeal cells. This trend has not been uniform, since the most highly developed eukaryotic cells have retained into the modern era multiple (linear!) chromosomes of more primordial design. These partly conflicting trends are not easily accommodated by a traditional phylogenetic tree, but as argued below (Sect. 7), the synthesis of Woese (1998) may let us view this tree in new perspectives.

In what has been called the Darwin–Eigen cycle (Poole et al. 1999), a positive feedback loop between increasing replication fidelity and large genome size allows new functions to be added to the cellular organization. Increased replication fidelity allows more genetic information, which could give a new cycle of improved replication. This starts at a rather low functional efficiency for all the initial components, but the initially high incidence of replication errors could rapidly expand the available sequence space for selective improvements. In turn, the average rate of replication errors has been further reduced, allowing more genetic information to be retained. Conceivably, much of the early phase of evolution occurred very close to the gradually increasing Eigen limit, only to be uncoupled from this limiting value

later on. What cues could be helpful in guiding our judgement as to when this uncoupling may have occurred, along the evolutionary ladder depicted in Fig. 1?

In terms of returns from the evolutionary investment, the overall fitness has likely increased with time in a roughly sigmoidal fashion¹⁸, somewhat resembling a classic growth curve of a microbial cell culture (the rather inefficient sequences of the beginning have much to gain, by risking little to lose; for the highly evolved sequences after extended periods of evolution, this relationship reverses and the risk of losing out by accidental mutation will exceed the potential chance of scoring further gains). The high chance for improvement during the quasi-exponential expansion at the beginning, therefore, creates a substantial “innovation pressure”, which keeps the residual error rate high and close to its limiting threshold. In other words, if any early protocell (or sequence) had shielded its mediocre information content behind the barrier of a relatively high fidelity of replication, this cell or sequence would have run a high risk of being outnumbered by mutant offspring of others that still exploited their higher innovation potential from more mutations. It was only when the sigmoid path of the cumulative fitness curve passed its point of inflexion¹⁹ that consolidation could take precedence over creating ever more novel sequences for further improvements. This must have been the turning point to biological speciation in the modern sense. Is this turning point best assumed to have occurred around the time of LUCA, or rather at a significantly earlier stage?

7

Woese's Phase Shift at Decreasing “Evolutionary Temperature”

According to Woese (1998), it is the likely state of LUCA itself that signifies this turning point. Instead of referring to Eigen's error threshold, he used a related term, the overall “evolutionary temperature”. This started out high and gradually diminished with time and protocellular consolidation²⁰. This indicator of innovation potential is composed of mutation rates and lateral exchange of sequence elements. As long as the “evolutionary temperature” was still high at the evolutionary time scale, the entire primordial gene pool was unified in a single universal lineage of potentially communicating protocells.

¹⁸ As yet, this is merely a conjectural assertion, unsubstantiated by rigorous modelling in mathematical terms. In the main, however, it follows from elementary considerations in that no autocatalytic (quasi-exponential) process in the real world can grow beyond all limits—together with the well-documented fact that modern organisms are optimized in their genetic outfit to such an extent that the vast majority of accidental mutations are now more or less detrimental.

¹⁹ The inflection point of a sigmoidal growth curve marks the transition from autocatalytic acceleration to progressive deceleration in approaching the limiting threshold.

²⁰ The related concept of *innovation sharing* was further explored for its potential of explaining the early evolution of the *genetic code* (Vetsigian et al. 2006).

Individual protocells need not have contained copies of all these genes at any one moment, but the cooperative members of coevolving biofilm communities, etc. could have done so as a group. Most members of this universal stem lineage may have retained a fragmented genome of multiple linear chromosomes.

These fundamental insights imply that interactive relationships of selective significance in the early biosphere were radically different from what we experience today. The main level of competition was still between replicating molecules—in whatever cytoplasm they happened to be supported—rather than between cellular organisms that had to defend their genetic identity. No gene-sized pieces could ever have existed entirely on their own. While individual sequence elements competed with related sequences for replication efficiency, they had to cooperate with many other (unrelated) sequence elements to create the supportive cytoplasm they all depended on²¹. The physical association of functionally interdependent replicators could be advanced at two different levels, by catenation into longer RNA/DNA molecules and/or the compartmentalization of groups of smaller pieces in a common envelope of protonuclei. While the first route requires the Eigen limit to the size of meaningful and heritable sequences to be raised by reducing error rates beforehand, the second route does not and therefore could have been followed early on. Accordingly, we are inclined to assume that the main stem lineage developed by nuclear compartmentalization—presumably in a plasmidial or syncytial organization with several nuclei in a common cytoplasm (Sect. 10)—before the catenation of fully self-sufficient chromosomes became ever more important.

On the Woesean view, the first separate lineage of extant organisms that effectively emancipated itself from the confines of the universal main line were the bacteria, which may have started out by particularly successful (circular) plasmids. In particular, such plasmids could have based their replication on a deviant processive DNA polymerase of the bacterial-specific C-type family (Koonin 2006)—in contrast to the prevailing B-type protein family employed by archaea and eukaryotes. These founding plasmids may have originated by gathering the genes for an entire pathway of metabolic significance²², but gradually adding more genes from other minichromosomes, until their information content had reached a sufficient size to establish fully functional cell lines under the direction of a single plasmid turned into a chromosome (Poole et al. 1999). The large Mimivirus (Suhre et al. 2005) has a linear genome about 1.2 Mb in size, carries many genes relating to general metabolism, and is a potential model for ancestral features at the hypothetical

²¹ This is another way of phrasing the essence of hypercycle theory, developed in mathematical terms to formalize the emergence of macromolecular cooperation (Eigen and Schuster 1977, 1982).

²² Although the potential significance for plasmids for early evolution has yet hardly be assessed, the plasmid-borne pathway of nitrogen fixation in rhizobial bacteria can serve as an extant example (Fischer 1994).

prokaryote–eukaryote transition phase. Eukaryotes appear to have retained multiple linear chromosomes, settling down with manageable compromises as to their size and number.

Another argument pointing at a virus/plasmid-related origin of the bacterial lineage concerns the peculiar problem of dimer resolution, which affects the faithful segregation of circular chromosomes; this is negligible for linear chromosomes as found in eukaryotes. Each single crossover²³ between partially replicated circular chromosomes creates a catenated dimer, which cannot as such be distributed to both daughter cells. Their faithful resolution depends on the cooperative action of a DNA translocase and a site-specific recombinase (Ip et al. 2003). Similar enzymes are also involved in conjugational transfer of certain plasmids and in prophage integration, respectively.

The great divide of the universal stem lineage may have coincided with the metabolic transition to carbon fixation by photosynthesis, which solely is a bacterial achievement. By its dependency on solar energy, this activity demanded the successful colonization of shallow waters and/or tidal flats, despite the accompanying environmental hazards, such as mutagenic UV light, etc. It required follow-up inventions to cope with the noxious properties of molecular oxygen—another bacterial contribution of evolutionary dimensions. The other protocells of the (no longer universal) stem lineage may have stayed in the sulfurous deeper ranges of the primordial oceans, but some of them may well have followed suit with the pioneering proteobacteria to the shallow flats. Recirculation of the newly opened resources of additional biomass was likely an irresistible incentive. Eventually, the remaining stem lineage must have split up once again, giving rise to archaea with a single (circular) chromosome per autonomous cell of minimal sizes, whereas the remaining lineage continued developing at higher intracellular complexity to the extant eukaryotes. In particular, the close association of members of the protoeukaryotic stem lineage with the oxygenic, photo-autotrophic bacterial mats has then facilitated the symbiotic internalization of protomitochondria, as well as plastids in the precursors to green plants.

The rationale of Woese's concept is modelled after more simple physical systems. In addition to *evolutionary temperature* and its *cooling* down, he frequently uses *crystallization* as a key metaphor²⁴. With this is meant the progressive association of interdependent components at different levels of both complexity and replaceability. At the originally rather loose fit between the components of any functional complex (e.g., a primitive ribosome at the subcellular level), the high primordial mutation rates would have furnished

²³ More generally, an uneven number of crossover events, which frequently occur during recombinational repair at stalled replication forks or other forms of DNA breakage.

²⁴ *Crystallization* is but one of several kinds of *phase transition* in the physical world (see Binder 1987). In a way, biologists' trials to look before the last common ancestor resembles cosmologists' attempts to grasp the various uncouplings that supposedly occurred in the immediate aftermath of the *big bang*—before the *cooling* and expanding universe became transparent to visible light.

replaceable spare parts from many sources, thus allowing for lateral gene transfer without significant penalties. As the fit became ever better with evolution inside a given lineage, it also became less and less advantageous to take in spare parts from a different lineage, and barriers were erected to restrain the indiscriminate transfer of genetic elements. This marks the emergence of *genealogical* identity—to use another one of Woese's terms.

On this view, the bacteria were the first entity to crystallize out as a recognizable lineage at the organismal level. Accordingly, these were also the first to deploy a high-quality, error proof replication system and erect barriers against excessive LTG from the non-bacterial main-stem lineage. It is not essential in the Woesean system that all lineages in *physical* existence developed their own *genealogical* identity simultaneously. The next group to crystallize out would then have been the archaea—still leaving behind a less-individualized main-stem lineage. It is from the latter one that the putative host for protomitochondrial endosymbiosis has been recruited, by which time—at the latest—also the eukaryotic lineage had gained its *genealogical* identity (Fig. 1).

Presumably, the prokaryotic domains of archaea and eubacteria have experienced regressive selection for metabolic specialization, genomic streamlining and size reduction²⁵. On the other hand, the phagotrophic mode of eukaryotes and their presumptive ancestors inherently required a higher level of complexity, not allowing miniaturization to the same extent. Genome-wide comparisons have shown that prokaryotes have experienced clustering of functionally related genes at a scale unseen in eukaryotes (Doolittle 2002). Plasmids have likely played a major role, both in the assembly of such clusters and in the lateral exchange of these clusters between different species.

Arguably the most important metabolic specialization of prokaryotes—as seen from the biased eukaryote perspective—has been the perfection of photosynthesis, accompanied by the oxygenation of the upper biosphere. This was well before the ascent of eukaryotic algae. Bacterial mats or biofilms in shallow waters were likely the most productive ecosystems during the long Precambrian era of unicellular life. It is reasonable to assume that amoeboid predators were present at that time, even though no truly premitochondrial amoebae are known to have survived into the modern world (Embley and Martin 2006). Shallow waters are most exposed to seasonal changes, especially during extended periods of global glaciation, which are apparent from the geological record. Moreover, when the pristine atmosphere was still essentially free of molecular oxygen, and/or ozone for that matter, shallow-water environments were unshielded from ultraviolet sunlight, which presumably resulted in much higher mutation rates in the exposed biofilms, as compared to the conditions prevailing today.

²⁵ An instructive example of gene losses in the early streamlining of prokaryotic genomes has been inferred from the distribution of ribosomal protein genes (Lecompte et al. 2002).

While these considerations on paleoecology are rather hypothetical by nature (see Labandeira 2005), they may be relevant for both the generation of organelle-bearing eukaryotic cells in the modern sense and the selection of meiotic activities from the very beginning (see below). The endosymbiotic capture of protomitochondria in the common eukaryotic ancestors, as well as photosynthesizing plastids in protoalgae, may have occurred in such environments (see Dyll et al. 2004). Mitochondrial cytochromes, in particular, have been shown to be related to those of ϵ -proteobacteria²⁶ (Baymann et al. 2004). At first, the endosymbiotic protomitochondria may have protected their host cells from oxidative damage by the accumulating oxygen, before they assumed their central role in energy metabolism later on.

The various complex specializations of eukaryotic cells, such as nuclear pore complexes, the mitotic spindle, the ER/Golgi network, etc., can likewise be conceived as having been established by *crystallization* processes in the Woesean sense. At the next level of complexity, also the stereotypic patterns of mitotic nuclear division and mainstream meiosis are two examples of this kind. In the following section we analyze the cooperative assembly of the meiotic system from various preformed components.

8

Early Traits with Preadaptive Value for Meiosis

The concept of *preadaptation* is a versatile device in the toolbox of evolutionary theory in general, especially in dealing with the establishment of complex morphological traits (Budd 2006). A certain feature is ascribed a *preadaptive* value if it is reused in a functional setup that is markedly different from its original role, especially if it is coupled with some degree of *redundancy*, such as duplication and subsequent specialization of participating genes. Meiosis, too, is a very complex trait, the origin of which is enigmatic. Here we discuss the original roles of four particular activities and their preadaptive potential for having been reutilized during meiosis²⁷.

- The cut-and-paste activities of topoisomerases.
- Recombinational break repair activities, including RecA-type recombinases.
- Mismatch repair activities involved in the repair of deviating single strands.
- The clustering of telomeres and their dragging across the nuclear envelope.

²⁶ as represented by *Aquifex aeolicus* among modern bacteria.

²⁷ At the other end of the spectrum, meiosis itself has been considered a *preadaptation* for the evolution of multicellularity (Maynard Smith and Szathmary 1995; see also D.-H. Lankenau, this BOOK).

The first three items are relevant for the initiation and performance of meiotic crossing-over, and the fourth feature may have led to homolog synapsis. The telomeric movements associated with the meiotic bouquet arrangement are of particular significance from the evolutionary perspective. This alternative mode of moving chromosomes may well have preceded the emergence of microtubule-based mitotic spindles as a primitive and less accurate mechanism of the segregation of chromosomes at nuclear division (Antoniacci and Skibbens 2006). According to this interpretation, the establishment of meiosis, too, would have started well before the mitotic system had been fully consolidated.

- (i) Topoisomerases are essential household enzymes of every living cell (Wang 2002). By breaking and resealing one strand or both strands of ds-DNA, topoisomerases of type I or type II can relieve excessive twist accumulating around active transcription or replication sites. In addition, type II enzymes²⁸ can resolve topological interlocking of DNA circles or loops, especially if the loops are constrained at their bases by binding protein complexes. Type II topoisomerases are functionally dimeric, with one catalytic subunit per DNA strand—often in tight association with additional subunits. Importantly, the catalytic tyrosine of the protein remains covalently linked to the 3'-phosphate at the broken end²⁹. This measure distinguishes the programmed cuts from indiscriminate damage; it also assures that the DNA breakage is readily reversible. Of ancient (pre-archaeal) origin, the catalytic subunit of topoisomerase VI has been reutilized as Spo11 in meiotic prophase of yeast and other eukaryotes to introduce DNA breaks at a limited number of sites. This programmed damage is then processed in a controlled and manageable way—introducing a certain number of reciprocal crossover events between homologs and repairing the remaining breaks without exchange (see S Keeney, this SERIES; G. Cromie and G.R. Smith, this BOOK). In most eukaryotes, Spo11 homologs are only expressed during meiosis, and the noncatalytic B subunit of the archaeal topo VI enzyme is missing altogether. In turn, a gyrase-type topoisomerase of bacterial origin (topo II) has likely replaced the original function of topo VI in vegetative cells. It is mainly in plants that one or two additional orthologs are present in the genome (see G.H. Jones and F.C.H. Franklin, this SERIES), including a functional gene for the noncatalytic B subunit. In *Arabidopsis*, SPO11-1 and SPO11-2 cooperate in meiotic recombination, whilst AtSPO11-3 is involved in DNA endo-reduplication in certain seedling tissues as part of the functional topo VI complex (Stacey et al. 2006). Notably, the ancient

²⁸ Type II topoisomerases, which are of special interest in the present context, are also referred to as DNA gyrases.

²⁹ Such covalent protein linkage after DNA cleavage is also observed for certain site-specific recombinases, such as prophage integrases or the FLP protein of yeast 2 μ plasmids (Grindley et al. 2006).

gene duplications giving rise to SPO11 paralogs have occurred before the diversification of extant eukaryotes (Malik et al. 2007).

- (ii) RecA-type recombinases and additional repair activities are present in essentially all living cells. Although they are not strictly essential at each cell division, their activities become crucial as soon as dsDNA is broken for internal or external reasons³⁰. The accurate recovery of the original sequence at a broken site is only warranted by recombinational processes involving an intact homologous sequence for templated neosynthesis around the break site³¹. Homologous recombination in mitotic DSB repair proceeds in several stages (Aylon et al. 2003; see J.E. Haber, this BOOK): 5'-end resection, formation of RecA-type helical filaments at 3'-ends, homology search and second-strand invasion, productive annealing and priming of templated DNA synthesis, reannealing of the neosynthesized strands of both ends, and closing of the remaining single-stranded gaps³². Eukaryotic RecA homologs are of Rad51 type in vegetative cells. The helical Rad51-DNA filaments have to be activated by Rad52 and other proteins.

In vegetative cells, the recombinational gap repair is preferentially targeted towards the sister chromatid, at least during the S phase and G₂. The transiently formed heteroduplex DNA between the priming 3'-end and the template is rather short-lived and rarely, if at all, converted to Holliday junctions, and most events are resolved without reciprocal crossing-over³³. In the meiotic crossover pathway, Rad51 is assisted by its meiosis-specific paralog Dmc1, sister chromatids are actively discriminated against as potential targets, heteroduplex formation is stabilized and driven towards single or double Holliday junction intermediates, and mismatch repair-related components are recruited in the resolution steps.

- (iii) Various mismatch repair (MMR) activities are present in most living cells. Again, these factors are not essential at each cell division, but they become important under mutagenic stress. Furthermore, MMR is effective in eliminating replication errors that have escaped proofreading of the replicative DNA polymerases, which has contributed significantly

³⁰ Internal causes include stalling replication forks or reactive radicals produced during oxidative respiration, whereas external causes include ionizing radiation or radio-mimicking mutagens, such as MMS (methylmethane sulfonate) or bleomycin.

³¹ As a last resort, unrepaired ends of DSBs can also be connected indiscriminately by "nonhomologous end joining", but this pathway usually results in small deletions and/or chromosomal rearrangements.

³² In technical terms, this mechanism is termed *double-ended SDSA* (synthesis-dependent strand-annealing), which was first proposed for transposon-induced gap repair in *Drosophila* (Nassif et al. 1994).

³³ In mitotic recombination upon DSB repair, the yield of reciprocal vs. nonreciprocal exchange events varies considerably with experimental conditions. Reciprocal mitotic crossing-over requires sufficient homology over 1.7 kb or more, whereas nonreciprocal gene conversion is quite efficient at ~250 bp of homology at both ends (Inbar et al. 2000).

to bring spontaneous mutation rates down to the present level. Two MMR-related proteins (Msh4–Msh5 heterodimers), in particular, have come under meiosis-specific control. Although they no longer are engaged in mismatch recognition directly, they now bind to Holliday junctions selectively, where they encircle both recombining chromatids (see J.E. Haber, this BOOK). Also, in mammalian meiosis Msh4 is specifically bound to recombination nodules, which are likely precursors of chiasma formation (see T. Ashley, this SERIES). Other MMR proteins actively participate in the processing of hybrid DNA in recombinational intermediates, where they critically contribute to the rejection of crossing-over between divergent sequences—duplicate genes at nonhomologous loci or homoeologous chromosomes in species hybridization (Surtees et al. 2004).

- (iv) A peculiar clustering of telomeres during meiotic prophase has long been observed in many organisms as the *bouquet arrangement* (Schertan 2007; see D.Q. Ding and Y. Hiraoka, this BOOK). As pairing and synapsis of homologous chromosomes often begins close to the telomeres, bouquet formation has traditionally been ascribed a supportive role for homologous partners to find one another. Structurally, the telomere clustering is based on the binding of telomeres to the inner membrane of the nuclear envelope, and the dynamics are dependent on cytoplasmic cytoskeleton components—generally actin filaments, but also cytoplasmic microtubules, varying with the organism. Characteristically, the coupling across the nuclear envelope is mediated by the LINC complex³⁴ (Crisp et al. 2006), which forms between SUN and KASH domain proteins in, respectively, the inner and outer membranes of the nuclear envelope.

By being attached to LINC complex proteins, the meiotic telomeres can yield to mechanical forces applied at the cytoplasmic side of the nuclear envelope—as another mode of moving chromosomes, alternative to the better known mitotic spindle (Chikashige et al. 2007). The active gathering of all the telomeres in a restricted area raises the chances of homolog encounters dramatically, as compared to random movements in three dimensions inside the entire lumen of the nucleus. Notably, two partly desynaptic mutants in *Zea mays* show telomere misplacement at the bouquet stage (Bass et al. 2003). Moreover, the widespread occurrence of the meiotic bouquet indicates that this alternative mechanism of chromosome motility, in fact, is of very ancient origin.

As we do not share the prevailing assumption that linear eukaryotic chromosomes with telomeres were secondarily derived from circular genophores as presently found in prokaryotes, we here suggest further modifications of

³⁴ Besides the meiotic bouquet, the LINC complex is involved in many other activities, such as nuclear positioning and migration, centrosome positioning, etc.

this scenario. A very interesting hypothesis on the evolution of eukaryotic chromosomes has been proposed by Villasante et al. (2007) that telomeres preceded centromeres in linking the chromosomes to cytoskeleton components. To our view, the active movement of telomeres by cytoplasmic forces that are transmitted across the nuclear envelope represents a very ancient mode of moving chromosomes, which most widespread has survived as bouquet formation in meiosis. The actual segregation of chromosomes—in both mitosis and meiosis—has since adopted an alternative mechanism based on centromeres and spindle microtubules.

This view has further implications. The main advantage of centromeres lies in the concentration of kinetic activity to a single site per chromosome³⁵, which guarantees the proper segregation of all the pairs of sister centromeres to the two spindle poles. For telomeres to fulfil a similar role during proto-mitotic division, both telomeres of each linear chromosome should act together as a functional unit, so that sister telomeres would disjoin coordinately at each division. This is formally similar to the requirements prevailing for the pairs of homologous centromeres at modern meiosis: respectively pairing/synapsis before meiosis I and disjunction of sisters at meiosis II. We are tempted to speculate, therefore, that certain key components for centromere interactions at modern meiosis were originally developed for telomere disjunction at a more primitive stage of proto-mitosis. In particular, this applies to synaptic pairing proteins to connect both telomeres³⁶, cohesion factors to connect the sister strands, and condensins to partly disentangle the looping sister chromatids. In addition, the coordinate segregation of telomere pairs would pull apart the chromatid loops, requiring further action of topoisomerases and/or recombinases for the resolution of interlocking connections between the strands. Such activities are likewise needed for the resolution of interlocks between internal chromatin loops of contemporary chromosomes.

9

Meiosis vs. Mitosis – Alternative Programs Responding to Different Selective Needs

The complex patterns of sexual propagation have rightly been called *The Masterpiece of Nature* (Bell 1982), of which meiosis constitutes the central hub. As pointed out in the more mechanistic chapters in this book or series, the functional relationship between meiosis-related mechanisms and mitotic

³⁵ The occasional occurrence of “holocentric” chromosomes, such as observed in various arthropods, is considered a secondary modification.

³⁶ The looping back of many minichromosomes by telomere-to-telomere attachment is, in fact, observed in macronuclei of ciliates (Postberg et al. 2001), which divide by an unconventional “amitotic” mechanism. Intranuclear microtubules are observed during macronuclear division, but their role remains to be determined (Tucker et al. 1980; Fuiju and Numata 2000).

components is still noticeable in many ways. It is indeed striking that similar components are used to drive two different chromosome segregation schemes (mitosis and meiosis). How can this dual strategy have been established and maintained by evolution?

Most authors addressing this question have been trained in higher animals, which are diploid, have separate sexes/genders (that is equivalent to being dioecious in botanical terms), and have their germline cells differentiated from somatic cells very early in development. Those factors alone can favor some limited or biased views, and most of the literature on the evolution of sexual reproduction is concerned with various aspects of the maintenance of meiotic sex in diploid organisms with separate genders, rather than preconditions for its original invention. The “cost of meiosis” handicap (also rephrased as “cost of males”), where half the genes are sacrificed in oogenesis and fertilization as compared to parthenogenesis (Williams 1975), would not apply to haploid organisms with zygotic meiosis, where all the products of meiosis on equal terms are contributing to the next generation. Accordingly, the “haploids first” hypothesis will be the key to further considerations regarding the ultimate origin of meiosis.

Generally speaking, the haploid way of eukaryotic life appears to be older than the diploid mode. Among the highly developed multicellular phyla, only animals are exclusively diploid. Flowering plants appear essentially diploid as well, but on the geological time scale this is a recent achievement; it has been derived from early green plants, such as the predominantly haploid mosses. The fungi are basically haploid organisms, and the same might be assumed for the common ancestor of all eukaryotes. If meiosis indeed has first arisen among essentially haploid unicellular organisms, certain arguments from a diploid perspective would no longer be relevant at that early stage.

In our view, it appears unlikely that a well-established, asexual, diploid organism would all of a sudden have “invented” the elaborate pattern of meiotic chromosome reduction (Penny 1985). It is easier to have a scenario of intermediary stages from the basically haploid level. It seems that meiosis developed in eukaryotic evolution before any one of the main phyla became multicellular, and so was probably ancestral to all eukaryotes (Ramesh et al. 2005).

In the world of free-living single cells, conditions often alternate between periods of ample nutrient supply and dwindling resources followed by stagnation—with a high risk for the population that all vegetative cells are dying from the harsh environment. These different external conditions exert opposing forces of selection pressure. At times of affluence, the winning choice is rapid growth and faithful replication (and because meiosis is a time-consuming process, it would be disadvantageous under such conditions). However, in times of deprivation, the goal is shifted towards dormancy as the safest option. For a single cell, the strategies of rapidly dividing or turning dormant are mutually exclusive, and the toggle switches to throw a given cell in either direction are tightly controlled. Accordingly, during the active

growing season, the alternative genetic program to attain the dormant state may be shut off for many cell divisions.

If transcriptionally inactive genes are impaired by mutation, then these potentially deleterious effects are not subject to immediate selection. Such mutations are expected to accumulate with time, especially if

- (i) rates of replication errors and/or environmental mutations are high and
- (ii) numerous genes contribute to a complex conditional system that are only expressed on rare occasions.

It has long been noticed, for instance, that commercial brewing strains of yeast, serially transferred from batch to batch in fermentation tanks, tend to lose the ability to sporulate compared to wine yeasts isolated from the wild. In experimental yeast populations, too, the same effect has been observed after extended selection for rapid vegetative growth (Zeyl et al. 2005). This inevitable trend to accumulate potentially deleterious mutations can be countered by intergenic complementation and/or recombination if two or more mutants of independent origin could re-establish the functioning of an alternative survival program. In the diction of Poole et al. (2003), the preparation for dormancy is a periodically selected function (PSF). Even in bacterial populations, such functions can be maintained by competence-mediated DNA uptake and lateral gene transfer.

The reasoning above is able to explain that meiosis in protists is preferentially linked to a dormant stage in the life cycle, such as the formation of hardy cysts or various kinds of endo- or exospores³⁷. If everything is going well, don't change your genetic combination; if things are going badly, recombination and meiosis may be the best option (Poole et al. 2003). If meiosis indeed has first occurred in haploid organisms, this means that its invention should have been preceded by some means of cellular fusion, which need not have been regular at the beginning. Such fusion could occur accidentally in a crowded biofilm, or it could result from active phagocytosis, as illustrated by amoebic slime molds (see below). Virus-induced fusion of crowded wall-less cells is yet another potential mechanism (Peisajovich and Shai 2003).

A cytoplasmic merger between two independent dormancy-deficient mutants allows the corresponding wild-type alleles to complement each other, and this immediate gain of function occurs irrespective of whether the nuclei fuse thereafter. Thus, complementation alone should give a selective advantage to any system of facilitated cytoplasmic fusion (syngamy) whenever such a cell population approaches a dormancy-inducing stage. Potential complementation between heterogenic nuclei in syncytial cysts is still widely observed in the ancient and successful group of Glomales (Pawlowska 2005)³⁸.

³⁷ Similar arguments have long been used to explain the alternation between sexual and asexual generations in facultatively asexual populations (see I. Schön, D.K. Lamatsch and K. Martens, this BOOK).

³⁸ These oomycete-related fungi form so-called arbuscular mycorrhiza in symbiotic association with plant roots, dating back to the earliest land plants in the fossil record. Incidentally, they probably have lost meiosis millions of years ago (see I. Schön, D.K. Lamatsch and K. Martens, this BOOK).

Moreover, if the nuclei fuse as well (karyogamy), genetic recombination can add to the advantage by re-establishing the dormancy-proficient genotype in part of the progeny—together with the reciprocal double mutants, which would be subject to selective elimination later on³⁹. The next problem is to envisage how the efficient system of a fully developed meiosis could have arisen in a series of likely and reasonable steps.

10

Coevolution of Meiosis and Mitosis

Coevolution of complex systems is a key concept in understanding the establishment of complementary traits by natural selection. More often than not this term is applied to inter-species interactions, such as adaptive predator/prey⁴⁰ or parasite–host relationships (Thrall et al. 2007); the more subtle interrelationship between flowering plants and pollinating animals is one of the most charming examples. Symbiotic systems are coevolving in many ways—down to the intracellular level of organelles (Rand et al. 2004). At the intraspecies level, too, coevolution is virtually omnipresent—be it among cellular and subcellular components, such as in the vertebrate immune system (Du Pasquier 1992), or among individuals, as evidenced by the widespread occurrence of sexual dimorphism, which is driven by mutual or antagonistic interests (Chapman 2006).

Given the power of coevolution from feeble beginnings, we consider it most likely that the evolutionary roots of proto-meiosis trace back to a stage when the segregational mechanism of proto-mitosis was not yet fully developed and stabilized. As argued above, the complementarity of mitosis and meiosis is best understood in terms of alternative needs at different times of the life cycle in a periodically changing environment. In this section we envisage a succession of stages in proto-mitotic nuclear divisions—in parallel with possible proto-meiotic advances from stage to stage.

In due course, genome organization changed from many gene-sized pieces to fewer chromosomes containing multiple genes, and chromosome movements during nuclear division changed from being telomere-directed and actin-driven to being dominated by centromere attachment to the spindle apparatus based on microtubules. We suspect that the primitive mode of moving many telomeres across the nuclear envelope was much less accurate than the centromere–spindle mechanism of a fully developed mitosis, thus

³⁹ Similar arguments have been put forward to explain the maintenance of meiosis in multicellular, diploid organisms (Kondrashov 1988; Crow 1994; see D.-H. Lankenau, this BOOK).

⁴⁰ The survival of the African megafauna provides an instructive example to the power of coevolution from a feeble start. In Africa, where the human species first arose, there was sufficient time for adaptation to the gradual perfection of human hunting skills—in contrast to Late Pleistocene extinctions on other continents, where human hunters of African descent arrived at a sophisticated level to which the biggest mammals could not adapt fast enough (Barnosky et al. 2004).

leading to frequent loss of chromosomes during division. The loss of chromosomes from individual nuclei could to some extent have been buffered in the population, if numerous nuclei stayed in a common cytoplasm for a considerable time, and if plasmodial fusion was not uncommon.

If the first steps towards proto-meiotic differentiation already started in the era of gene-sized minichromosomes, reciprocal exchange by crossing-over may have been less important than it is in contemporary meiosis. This is because the need of recombinants for different genes could largely have been satisfied by the reshuffling of independent minichromosomes. Hence, in our conceptual attempts to disentangle the high complexity of meiosis into a likely series of minor steps for its origination, some primitive means of ploidy reduction after nuclear fusion could have come first, whereas the widespread occurrence of chiasmata only started later on. If the segregation system could just about handle a haploid set of many minichromosomes, it might have been overburdened by the doubling of chromosome number after occasional karyogamy. This would result in a substantial load of aneuploid nuclei being produced in the divisions following karyogamy—until a balanced haploid set was re-established by the successive loss of chromosomes.

It has long been noted that the mitotic segregation of chromosomes tends to get unstable when cells of preferentially haploid organisms are deliberately maintained at the diploid level. This has led to so-called parasexual procedures of genetic analysis, as pioneered for mold-like fungi (Clutterbuck 1992) and further developed for fission yeast (Kohli et al. 1977) and amoebic slime molds (King and Insall 2006). On rare occasions, diploid cells of such organisms can arise spontaneously, or they can be procured by artificial cell fusion techniques in the laboratory. Although these diploid cells are reasonably stable, they tend to lose single chromosomes at random at a measurable rate per cell division⁴¹. The resulting monosomic cell lines, however, grow less vigorously, if at all—giving rise to variable aneuploid subclones and lethal sectors⁴². It is only after a balanced genome has been re-established at the haploid level, by the successive loss of other chromosomes, that maximum growth rate and mitotic stability are being regained in such a cell culture. It is not unreasonable to assume that similar selective trends have been significant to the establishment of meiosis in early eukaryotes.

As briefly noted above (Sect. 8), we suggest that telomere pairing within each minichromosome may have been recruited into proto-meiotic differentiation as the structural basis for homolog pairing and synapsis. As surmised for the proto-mitotic segregation mechanism, both telomeres of each duplicated minichromosome should conjoin for coordinate disjunction. This preferential conjunction may be upset after karyogamy, since the telomeres of the homolog would compete with the other telomere of the same chromosome for

⁴¹ The rate of chromosome loss can rise further by exposure to environmental spindle poisons.

⁴² A subclone started by a defective cell where all the residual divisions eventually abort is often referred to as a “lethal sector”.

pairwise binding. The ensuing instability might be alleviated by a step-wise differentiation between both kinds of pairing—as in contemporary meiosis—by first disjoining the homologs, and the sister chromatids thereafter. The customary suppression of DNA replication between both rounds of chromatid segregation may originally have started as a checkpoint response against the segregation instability, which subsequently became reinforced genetically and integrated in the modified meiotic cell cycle.

How then can recombination be introduced in some intermediate role to drive the evolutionary trend of coupling the proto-meiotic cell-cycle modification with preparation for the dormant state? In fact, recombination between differently mutated, homologous chromosomes would gain significance with the increasing number of genes per chromosome.

One way of using non-isogenic DNA could be the unidirectional integration of smaller fragments by some process of gene conversion, as opposed to reciprocal exchanges between entire chromosomes. Spore-forming bacteria, such as *Bacillus subtilis*, are known to do so regularly by acquiring the competence for transformation in parallel with sporulation (Grossman 1995; Lazzera 2000). Upon starvation, these spores are formed internally by asymmetric compartmentalization of the mother cell. The spores are liberated by lysis of the mother cell; concomitantly, the DNA equivalent of an entire genome is discharged to the environment. Other cells in a starving population do not sporulate themselves, but they enter a state of “competence” to actively internalize single-stranded pieces of DNA for local recombination into the chromosome. The early phases of differentiation towards either competence or sporulation are partly coordinated by a common set of genes. Again, in the words of Poole et al. (2003), the maintenance of bacterial sporulation and uptake mechanisms of transforming DNA would be subject to PSF-type selection. Yet, in the setting of plasmodial, amoeboid cells, which already had developed a proto-meiotic fusion-disjunction cycle, a stage of unidirectional recombination—using fragmented DNA from partially deteriorated nuclei—may not have added such a great advantage.

The widely present Spo11 homologs in the initiation of meiotic crossing-over suggest a different evolutionary path. As these proteins represent the catalytic subunit of a former DNA topoisomerase VI, proto-meiotic crossovers (at first) may merely have arisen as side products of a deranged topoisomerase reaction. Usually, of course, the closing of DNA molecules occurs in situ between the same strands that just have been cut by the (dimeric) topoisomerase—after an interlocking DNA duplex has been granted passage through the temporary gap. The resealing in situ depends upon the dimeric subunits staying in physical contact all the time⁴³. Incidentally, if their contacts are broken prematurely, the two ends of the double-stranded

⁴³ During the topoisomerase II reaction, the point of inter-subunit contacts changes from one side of the duplex to the other—similar to the top and bottom gates of a lock in a shipping canal.

break (DSB) may drift apart. This, in turn, alerts a DNA damage response in order to seal the break by the ubiquitous recombinational repair pathway. If the homolog is chosen as a template for the repair synthesis required in this reaction, this adds a significant advantage to the proto-meiotic disjunctive mechanism in that it stabilizes the interacting molecules before segregation—especially in the advanced mitotic mechanism of centromere–spindle attachments. In addition, it increases the possibilities of recombinant progenies for subsequent selection—especially as the number of genes per chromosome has risen with time.

11

Variations on the Meiotic System in the World of Protists

11.1

Fission Yeast as a Haploid Model Organism: Zygotic Meiosis Before Sporulation

The fission yeast *Schizosaccharomyces pombe* has long been used to study cell-cycle controls, as well as meiosis and sporulation as alternative developmental programs (Nurse 1990; Egel 2000; G. Cromie and G.R. Smith, this BOOK). In this yeast, the fusion nucleus in a zygote is the only diploid stage in the life cycle, and the four nuclei after meiosis II are encapsulated in individual ascospores, each one of which is able to survive and start a new yeast population upon spore germination. Although this organism is quite simple, it is not “primitive”, since it is assumed to have undergone simplification to unicellular growth by evolutionary regression from more complex filamentous fungi (Sipiczki 2000). Only the haploid state of nuclei during vegetative growth is considered to be ancestral throughout the fungal kingdom and beyond.

The sequencing of the genome of *S. pombe* has allowed genome-wide expressional analyses. In particular, the alternative programs related to sexual fusion, meiosis, and sporulation affected some 1000 genes by substantial up-regulation during the course from nitrogen starvation through pheromone response, meiosis, and sporulation (Mata et al. 2002). Comprising about 20% of the entire genome, this is a sizeable fraction of the overall coding capacity; equivalent results were also obtained for baker's yeast⁴⁴ (Chu et al. 1998). Most of these genes do not directly affect the efficiency of vegetative growth and mitotic cell division, but defective alleles can reduce the ability to sporulate, or even abolish it completely (Yamamoto et al. 1997). These genes therefore represent a complex conditional system of alternative expression, as discussed above, which may have driven the establishment and maintenance of meiotic recombination.

⁴⁴ Budding yeast meiosis is given less attention in this section since the mainly diploid life cycle is certainly a derived feature in the fungal lineage.

Moreover, the importance of ascospore formation for the ecological fitness of yeasts is indicated by the independent development of switching homothallic mating-types in both budding and fission yeasts. In contrast to mold-like fungi, the unicellular yeasts cannot form dormant vegetative spores (conidia); the sexually generated ascospores are their only means of long-term survival in a natural environment. This presents a problem when a suitable mating partner is not available, such as in a droplet of plant-derived sap or juice that has been inoculated by a single yeast spore. Yet, such very suitable microenvironments for yeasts are rapidly exploited and then the starving yeast cells are subject to being fed upon by snails or fruit flies. Only the ascospores can resist the digestive enzymes in the gut of these predators. To give populations derived from single ascospores a chance to complete the cycle, again producing ascospores, both budding and fission yeasts have intricate systems of mating-type interconversion.

The molecular switching mechanisms in budding and fission yeasts are similar in their effects, but different in about every detail (Haber 1992; Egel 2005). The comparable aspects are as follows. Partly redundant mating-type information occurs in the genome as two complementary, transcriptionally silenced storage cassettes. In certain cells, before division, a copy is mobilized from one of these silent loci and transposed into the expressible site, the active mating-type locus. In consequence, one or both daughter cells after division will then express the opposite mating type to the mother cell. To initiate the actual switching event, a programmed damage is inflicted on one or both strands at the resident cassette of the active locus. This genetic damage is subsequently repaired by the pathway of homologous recombination, as guided by flanking stretches of perfect homology at all three loci. With a high degree of preference, the silent cassette bearing the opposite mating type of the residing active cassette is chosen as a template for repair by “synthesis dependent strand annealing” through the donor cassette (see Figs. 14/17C of J.E. Haber, this BOOK). This bypassing synthesis is then resolved at the other end of the active site—again within a stretch of perfect homology that is present at all three loci.

It is remarkable indeed that the two yeasts have independently reached very similar mechanisms. Certain aspects of this evolutionary trend even resemble the establishment of meiosis in the first place. Both meiosis in general, and the yeast mating-type switch, aim at homologous recombination on a regular basis. As the corresponding recombinases belonged to the universal toolbox of any cell—to deal with the ubiquitous repair of externally inflicted or replication-related double-strand breaks in the genomic DNA—it was possible, and relatively straightforward, to trap these enzymes into action by the deliberate placement of programmed genetic damage at appropriate sites by the cell itself. Given that rather inefficient results of such recombinational repair may have contributed little by little to positive feedback loops, they could in turn be optimized by repetitive selection and have led to very remarkable phenomena indeed.

11.2

Amoebic Slime Molds: Formation of Cannibalistic Zygotes

The cellular slime mold, or social amoeba, *Dictyostelium discoideum* has long been studied for its nonsexual mode of aggregation and differentiation, but its less easily observed sexual mode also has interesting characteristics. In particular, a delicate balance can be observed between sex and cannibalism in the course of so-called macrocyst formation. The haploid amoebic cells thrive on bacteria until the supplies get scarce and the population reaches a critical density. Then many of the free-living cells aggregate in preparation for dormancy. In this case, there are three ways of becoming dormant—"microcysts" are formed by individual cells; stalked "sorocarps" with many spores require cooperation of many vegetative cells; and "macrocysts" are initiated by zygote formation, likewise amidst an aggregate of many cells. The degree of dormancy for the three different resting stages increases in this order, macrocysts being most resistant. Characteristically, the sorocarps tend to maximize exposure to the open environment for rapid spreading, whereas the macrocysts are formed secluded in the dark and facilitate long-term survival through the winter season.

Of particular interest, the two modes of social aggregation are only observed in crowded populations, and the formation of sorocarps or macrocysts is started by rather few cells as a preemptive strike of differentiation. These cells act differently to most surrounding cells, and in so doing they organize the formation of aggregation centers, while the formation of competing centers in the vicinity appears to be inhibited.

Experimental research in *Dictyostelium* has focused on sorocarp formation, where haploid asexual spores are formed at the tip of a slender stalk. The initial aggregation of amoeboid cells is organized by a concentric wave of cAMP secretion and chemotactic movement towards the center of the gradient (Fontana et al. 1986). The cells that start this wave remain at the center, and they are predestined to form the spores at a later stage (Huang et al. 1997). These cells are also characterized as being starved in late-G₂ phase of the cell cycle, and they concentrate at the tip of the early mound (Araki et al. 1997). The fully assembled aggregation of $\sim 10^5$ cells then starts to move collectively in a slug-like fashion, eventually coming to a halt with a rising tip. The rear and inner mass of dying cells then contributes to the supportive stalk. Only the leading outer cells collect at the very top and differentiate into a blob of living and durable spores (Weijer 2004).

Sexual fusion of two amoebal cells occurs more rarely, but once a zygote has been formed it starts to attract surrounding amoebae, which simultaneously become inhibited in zygote formation on their own. A tightly packed lump of several hundred amoebae become ensheathed in a progressively thickening wall. The giant cell at the center, which is the original zygote, begins engulfing other cells around it, and does not stop before it is the only

cell remaining in the maturing macrocyst (Saga et al. 1983; Ishida et al. 2005). Meiosis eventually occurs when dormancy is broken at spring time, upon germination of the macrocyst. The post-meiotic nuclei segregate to haploid amoebal cells, which thereby reiterate the life cycle.

Contemporary slime molds are certainly not ancestral eukaryotes! We do not even know whether amoebae or flagellates were the earliest eukaryotes to set the stage for meiosis. What we do appreciate about the amoebic state and the engulfment of cellular food particles is the opportunity this offers for the development of pre-existing food receptors in the membrane into novel receptors for recognition of potential mating partners, such as the pheromone receptors thoroughly studied in yeast (Hoffman 2005). The difference between cannibalistic phagocytosis and zygote formation lies only two membranes apart. If both membranes remain intact, the fully engulfed cell ends in a lysosome to be digested. If the membranes fuse, however, the cytoplasm merge and the nuclei become available for further interactions. For reasons outlined before it should be advantageous to exchange genetic information at that stage of the life cycle. On the other hand, the first hapazard encounters of two nuclei with centrosomes and sets of chromosomes in a common cytoplasm must have been inherently unstable, and the risk of erratic segregation would increase with the number of chromosomes.

The so-called plasmodial slime molds, such as *Physarum polycephalum*⁴⁵, are not closely related to the cellular slime molds mentioned before. Their syncytial development could provide additional insights, but the sexual aspects of the life cycle in such species are little studied yet. In the context of the above discussion, it is worth noting that the haploid amoebae of *Physarum* can fuse and dissociate quite freely, irrespective of complementary mating types (Bailey et al. 1990). Successful karyogamy, however—as well as the subsequent establishment of multinuclear plasmodia—requires the hetero-allelic complementation from different nuclei. This illustrates that membrane fusion in the amoebal state has not necessarily been the limiting parameter in the primordial establishment of a sexual life cycle in early eukaryotes.

12

Concluding Remarks

It is argued above that meiosis developed as a successful solution from the disruptive instability of early quasi-sexual encounters, reusing most of the mitotic functions and modifying others. After all, meiosis is not only a stage of recombination; it is also a mechanism for returning to the haploid state in a regular and efficient manner. The efficiency of how both these aspects are

⁴⁵ The *Physarum* life cycle starts with haploid spores formed after meiosis, which germinate as haploid amoeba. The macroscopically visible plasmodia, containing many diploid nuclei, develop from zygotes formed by the fusion of sexually compatible haploid amoebae.

tightly interwoven in meiotic cells of contemporary organisms calls upon our admiration. In the primordial era of fragmented genomes comprising many gene-sized pieces or minichromosomes, the combinatorial aspects of proto-meiosis may to a large extent have been satisfied by independent minichromosome assortment, rather than molecular recombination of the DNA. In this scenario, the reduction of ploidy after incidental karyogamy may have preceded the incorporation of crossing-over and chiasma formation in the meiotic repertoire.

As far as we can see, most of the subprocesses making up meiosis are advantageous in their own right. They include making and reforming DSBs in DNA by topoisomerases, local gene conversion and reciprocal exchanges of longer chromosome sections, together with nuclear fusion and re-assortment of chromosomes during a parasexual cycle (which requires a mechanism for halving chromosome number after incidental diploidization). In this sense, meiosis is less of a mystery if all the steps are advantageous, even if this does not give any detailed picture in which order important steps were coordinated into meiosis as we know it.

It is reasoned further that the major selective benefit of fertilization and/or meiotic recombination in predominantly haploid, unicellular organisms is linked to both the short-term quenching of conditionally deleterious mutations in complementing zygotes and the facilitated elimination of multiple mutations of this kind among the haploid progenies. This periodic selection affects the subset of conditionally vital genes that is involved in a rarely used alternative survival program, such as the shift from active metabolism during vegetative growth to the dormant state of cysts or spores⁴⁶. In multicellular and often diploid organisms, most specialized functions of differentiated body cells would likewise fall in this category of alternative programs, in contrast with the basic household functions necessary in every cell. Thereby, together with bacterial competence and sporulation, meiosis can be considered the most prominent example of PSF-type selection—the maintenance of periodically selected functions (Poole et al. 2003).

In the eukaryotes-early scenario, as favored here for the main stem lineage emerging from the LUCA era, the development of a full complement of proto-meiotic functions may already have taken place in the innovative period when the prevailing error rates were still significantly higher than in the organisms we know today—though still below the limiting Eigen threshold. If the main stem lineage of that era (as we surmise) still carried many gene-sized pieces or minichromosomes, the corresponding Eigen threshold

⁴⁶ According to another (frequently repeated) argument in association with the formation of dormant spores or seeds, the major advantage of meiosis is ascribed to the generation of genetically diverse offspring for better fitness in an unpredictable environment (e.g., Bürger 1999). While we do not deny any ancillary effect of such a contribution, we think that repetitive selection by predictable changes (seasonal environmental bottlenecks and mutational load on periodically selected genes) is more likely to have driven the early coevolution of mitosis and meiosis as discussed above, than the less tangible response to unpredictability as implied by the alternative suggestion.

could still be considerably higher than required for the maintenance of bacterial chromosomes. The PSF-type selection for a dormancy stage and its meiotic protection may have set in when the early protoeukaryotic organisms followed the early photosynthesizing bacteria to the environmentally exposed shallow reaches of the primordial oceans.

We have probably not given the expected answer on the “origin” and “evolution” of meiosis. The standard assumption appears to be that meiosis was something “added on” after eukaryotes were well-established. That is, after the organization of mitosis, linear chromosomes, DNA repair enzymes, etc., had been fully accomplished. In a sense we are reversing the standard answer and suggesting that some simple form of cellular self-recognition, recombination, and repair mechanisms all existed comparatively early in the origin of life—at least as far back as the ribo-nucleoprotein (RNP) world, and possibly as far back as the RNA-world. We call this the exchange/segregational coevolution hypothesis for the origin of meiosis, in that some simple form of the overall processes occurred very early in cellular evolution of haploids, and that the many subprocesses just kept improving (“coevolving”) along with mitosis, DNA repair, and cellular recognition of self and of prey.

In this model, the earliest roots of meiosis can be traced back to the premitotic era when genealogical identities of different lineages had not yet been perfected and the sharing of information from compatible sources was of similar importance as the protection of a given genome. This led to a bimodal life-cycle strategy of alternating division schemes, where mitosis perfected the identical reduplication of the momentarily fittest genome, whilst meiosis—once per life cycle—revived and formalized the sharing potential of earlier lineages, as driven by the need to maintain a set of periodically selected functions. At first, the meiotic alternative occurred primarily by reassortment of many minichromosomes. Later on, as individual chromosomes grew larger, and their number per genome was reduced accordingly, molecular recombination of chromosomal DNA became ever more important for the meiotic program of genome sharing. This led to the recruitment of preexisting recombinational repair mechanism into the fully operational meiotic program, which is common to essentially all contemporary eukaryotic lineages.

Some simple mechanisms for recombination and repair mechanisms had existed extremely early, but they continued to increase in efficiency during the expansion in complexity of protein synthesis and the takeover by DNA as the primary coding molecules improved. It is worth going back to continually improving replication fidelity in Fig. 1. There is no “sudden” invention of the complex phenomena of genetic replication or recombination—just a continuing improvement over time. Similarly, meiosis was never “invented” en bloc either—and only after the eukaryotes had reached their full cellular complexity. Coevolution may also have reinforced a seesaw cycle shifting between alternative modes of genome segregation, in response to cyclic environmental changes, which eventually resulted in meiosis vs. mitosis as we know them today.

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The Legacy of the Germ Line – Maintaining Sex and Life in *Metazoans*: Cognitive Roots of the Concept of Hierarchical Selection

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Abstract The metazoan germ line is often referred to as nothing else but another organ or tissue that needs no different treatment in research compared to other somatic organs such as liver, kidneys, skin, brain or blood. However, the germ-line concept was established during the 19th century by August Weismann and was recognized since to be tightly linked with the role of maintaining sexual reproduction. By far more profoundly, Stephen Gould only recently documented how August Weismann and Charles Darwin gained insight into evolution driven by multiple levels of selection—not natural selection as the only level, each playing its particular and significant role in understanding a different aspect of evolution from the level of molecules to cells, man, societies up to the complexity of ecosystems. This chapter represents a single argument pinpointing the historical and current cognitive foundations at the grass-roots level with links to present concepts of sexual reproduction. Feeding back on this, sexual reproduction and genetic recombination including meiosis are part of the glue linking all the levels of hierarchical selection. Finally, this essay briefly sketches a tie to modern frontiers, in particular to the *nuage* germ plasm organelle. The *nuage* is recognized as an ancient key platform mediating genome encoded sex-related developmental circuits as well as germ-line-related control of transposon driven (epi)genome instability.

Abbreviations

HJ	Holliday Junction
LUCA	last universal common ancestor
miRNA	microRNA
PGC	primordial germ cell
pPGC	presumptive PGC
SDSA	synthesis-dependent strand annealing
the three Rs	recombination, repair, replication

1

Introduction

The human organism is multicellular and *Homo sapiens* thus belongs to the metazoan clade. On a superficial level, it is only natural to consider a special interest in our own biology. On much deeper grounds, and based on

mathematical and physical cognition, it is utterly crucial to grasp and penetrate the most fundamental biological circuits of life in order to feed back on future-oriented practical clinical approaches such as stem-cell-assisted gene therapy or tissue transplants. At the heart of these concerns lies the conceptual recognition of how multicellular organisms evolved in deep past from a single-celled root, and in contemporary ontogeny from the fusion of two germ-line members called gametes to an adult individual—being made up of mortal somatic cells and potentially immortal germ-line cells. The depths of significance of the differentiation of soma cells from germ-line cells were first elaborated by August Weismann. His perception at this point transcends much further beyond current medical importance to the levels of society formation and ecological systems. The legacy of the germ-line concept as a real biological entity, recognizable by germ-line-specific molecular markers ties meiosis, recombination and the maintenance of sex throughout deep time. The germ-line signatures such as meiotic recombination belong to a world, full of complex and still unsolved molecular phenomena¹. Prior to meiosis, DNA damage and repair, and the activity of transposons rule recombination relevant for genetic inheritance. Meiosis escorts these processes during a later developmental stage, but the relative quantitative importance on recombination of all three activities is scarcely known. During meiosis, a special enzymatic machinery is deployed to break or “damage” DNA “on purpose” (S. Keeney, this SERIES). The programmed lesions are then processed by substantial recombinational repair events and follow-up activities, such as inhibitory measures between sister strands and preferential interactions between homologous chromosomes instead (K. Tanaka and Y. Watanabe, this SERIES). Extending these means, meiosis is widely believed not only to merely halve diploid genomes but by its recombinational power to regulate elimination of detrimental mutations and to fix and spread advantageous mutations within natural populations. The validity of this latter key assumption, however, only recently has become challenged. Other processes such as transposon activities or replicative DNA damage repair and associated ameiotic recombination may complement meiotic recombination in asexual species (Omilian et al. 2006; I. Schön, D.K. Lamatsch and K. Martens, this BOOK). As the meiotic recombinational enzyme machinery is likely derived from more ancient DNA-repair pathways (R. Egel and D. Penny, this BOOK), and because DNA damage occurs continuously (Friedberg et al. 1995; Kaneko et al. 1996) at any time during germ-line development, an unanswered but most significant question arises from these considerations: how is the relative importance of meiotic recombination weighted against DNA-damage repair, mitotic recombination and replication (the three Rs) and against the activity of transposable elements (i.e., the most effective, and ancient recombination tool boxes) in the germ line? Is there a link to the *nuage* germ plasm organelle

¹ see Box 1: “Germ-Line Signatures and Cytoplasmic Means”

(Box 1)? Are there conserved germ-line-specific signatures such as epigenomic tags influencing the pathway chosen for DNA damage repair? Are there recombination-related links between chromosome territoriality (Johnstone et al. 2005; Knaut et al. 2000; Snee and MacDonald 2004; Tanabe et al. 2002) or heterochromatin and the germ line versus soma, as heterochromatin had been suggested to play some role in “germ-line-related functions” in the past (Hennig 1986)? The latter question actually is not as far-fetched as it seems: Energetically excited nucleic acids can return at a rate of femtoseconds into their ground state or can react fast with other chemical reactants. Physically, DNA bases therefore can function as a sort of “sunblocker” (Bhattacharjee 2001; Pecourt et al. 2000). Even though eukaryotic cells endure about 10 000 DNA strand breaks every day, a sunblocker-feature of heterochromatin perhaps represents an effective local protection against genetic damage. These questions have only recently become tackled and remain controversial, e.g., with evidence that sun-blocker protection is not taking place on a territorial level (Gazave et al. 2005). Other data, however, point towards differential relative use of different DNA repair pathways during germ-cell development (Engels et al. 2007; Johnson-Schlitz et al. 2007) and appear highly significant for future exploration. To tackle these questions it will be necessary to employ systems biology’s fittest and thoroughly established metazoan model organisms such as the fruit fly *Drosophila*, the nematode worm *Caenorhabditis*, the zebrafish *Danio* and the mouse *Mus*. The scope of this essay, however, is not to cover these systems. Recent public debates relevant to human genetics (Chong et al. 2007; Horstehmke 2007; Suter and Martin 2007) reflect only the tip of the iceberg of the beginning of a long scientific journey. The present chapter is thought to explore the grass-roots level of currently arising questions as to how the relative impact of the three Rs in relation to meiosis and transposon activity affect the genomes of somatic and germ-line cells, whole organisms and finally populations with a transspecific (i.e., macroevolutionary) flavor. The cognitive roots of recognizing that selection acts on multiple levels unifies these approaches and causally links the legacy of the germ line with maintaining sex in metazoan species and beyond. Let us travel to the foundations more than a hundred years ago and get a taste of how the first steps into a new frontier of scientific cognition and endeavor were taken.

2

The Legacy of the Germ Line

2.1

Germ Line: Definitions

There is actually no decent explanation for why on the one hand the items *germ*, *germ cell*, *germ plasm* and *germinal* are well defined in Webster’s New

Encyclopedic Dictionary and in the Compact Oxford English Dictionary, while on the other hand the term *germ line* is ignored in these most authoritative English glossaries. One might object that these are abridged dictionary versions, however, the term *germ line* is definitely superior compared to the listed terms. *Germ line* is not a very mundane expression, and that is why it is important to make sure that we understand it right. Let's begin with the definitions of the alleged inferior terms: **Germ** is defined as a portion of an organism capable of developing into a new one (Compact Oxford English Dictionary). Webster's Encyclopedic Dictionary defines **germ** as a small mass of living substance capable of developing into an organism or one of its parts. **Germ cell** is defined as an egg or sperm or one of the cells from which they arise (Webster) or a gamete, or an embryonic cell with the potential of developing into one (Oxford). **Germ plasm** – a term already used by August Weismann (see below) is defined as germ cells viewed as

1. the bearers of hereditary material or
2. as genes (Webster).

Germinal means of or relating to a germ or germ cell (Webster). It is difficult to imagine that these definitions help school kids, legal practitioners, or the average lay person to deduce the deep meaning of *germ line*. For that reason possibly my own school edition of the Advanced Learner's Dictionary (Oxford, 1963) just skipped all of the terms from its content, except *germ*. The difficulty of the subject, i.e., to exactly grasp what *germ line* is about may perhaps best explain why trimmed dictionary versions as a rule abandon the option to mention *germ line*.

Nevertheless, a full version of Webster's Dictionary² defines: *germ line* or *germ track*: *the sequence of cells from zygote to functional germ cell*: **GERM PLASM**.

Even though this definition is quite easy to comprehend and even though it is part of the unabridged Webster's Dictionary it is still surprisingly incomplete. Somehow, here the term *germ plasm* and the item *germ line* (though quite correctly defined) overlap and seemingly appear not to be as sharply separated as expected—and in fact the definitions of these items may be misunderstood to include some sort of misconception. As the matter is sometimes discussed controversially even among scientists the more difficult it appears to be for a layperson to shed light into this jungle of important topics. However, as a justification, the reason for some of the difficulties seems to be partially based on grounds of truly important discoveries in past history itself, encompassing ideas from intellectual giants like Charles Darwin and August Weismann, which nowadays have gotten intermingled with modern concepts of molecular biology, genetics, epigenetics, biochemistry and the modern synthesis of evolution. One aspect in Webster's above definition

² Webster's Third New International Dictionary OF THE ENGLISH LANGUAGE UNABRIDGED, 1992 Merriam-Webster ISBN 3-8290-5292-8 Könnemann Verlagsgesellschaft mbH

of *germ line* indeed is clearly neglected: it is not only the sequence of cells including the germ plasm (i.e., genes) from zygote to functional germ cell but it is by far more the transport of the genetic information encoded within the chromosomes, genes, germ plasm, DNA or how you wish to name it into the next generation. And this information transfer perpetuates into deep time including many thousands and thousands of generations of creatures (Fig. 3). It actually is the interface where experimental biology and historical biology (paleontology) meet.

Fortunately, at last I found one dictionary-entry with an acceptable definition in Merriam-Webster OnLine:³ ***germ line: the cellular lineage of a sexually reproducing organism from which eggs and sperm are derived; also: the genetic material contained in this cellular lineage which can be passed to the next generation.***

The other important term, *germ plasm*, was used by Weismann originally the same way we would use the terms *gene* or *genetic material* or DNA today. This is why Merriam-Webster OnLine defines:

germ plasm:

1. *germ cells and their precursors serving as the bearers of heredity material and being fundamentally independent of other cells*
2. *the hereditary material of the germ cells: GENES.*

However, this definition today is still handled controversially in the scientific community because the maternal cytoplasmic contributions deposited in the oocyte as well as extranuclear paternal material (i.e., centrosomes, mitochondria, protein complexes etc.) pass into the next generation and could play epigenetic roles in determining which post-zygotic cell becomes a germ-line cell in the early embryo⁴ (see also Box 1). Epigenetic modifications of chromatin proteins have been shown to be passed on to the next generation via the germ line (Cavalli and Paro 1998). In this contemporary context, Tobler (Tobler 1986) and others (e.g., Strome and Lehmann 2007) are using a definition of *germ plasm* different from Weismann's (see also Eddy 1975): "... a substance present in the cytoplasm of gametes,⁵ which is segregated into specific cells (during blastulation) and determines that those cells shall become the 'progenitors of the germ cell line' (= PGCs) during subsequent development".

Making this introduction even more perplexing—we here have to ask: what does this all have to do with maintaining sex in multicellular organisms over geological periods of time? It has by definition! But to demonstrate this requires quite a number of interdisciplinary leaps between scientific disciplines—historical, cell biological, field biological, population biological and genetical.

³ <http://www.merriam-webster.com>

⁴ http://www.nobelprize.org/nobel_prizes/medicine/laureates/1995/nusslein-volhard-lecture.html

⁵ Tobler mentions oocytes only but I prefer to include sperm as well, as some taxa (e.g., *Diptera*) incorporate entire sperm tails into the cytoplasm of the zygote (Karr 1991). Also, centrosomal proteins appear to be provided from sperm in many other species.

2.2

The Continuity of Weismann's Germ Plasm and the Theory of Inheritance

In 1674 with van Leeuwenhoek's microscope discoveries of microbial animalcules and in 1766 with Spallanzani's experimental proof of the division of a single animalcule⁶ (de Kruif 1926) natural history (i.e., biology) entered the race for understanding biodiversity. After Darwin had published his most influential works, i.e., "*On the Origin of Species*" and "*The Descent of Man and Selection in Relation to Sex*" (Darwin 1859, 1871) selection was progressively more recognized as the driving mechanism of evolution. The major driving force in evolutionary research was the question as to how selection could influence the morphological characters, and physiological traits of organisms of all shades (Mayr 1963). Since August Weismann's insights (Weismann 1892, 1893), the awareness of the existence of a *germ line* in multicellular organisms did play a significant, unifying and leading role in this.

The homologous body parts of animals differ taxon to taxon in their organizations, sizes, shapes and capabilities. Asymmetric cell division partially controlled by asymmetrically localized components of a specialized type of cytoplasm, the *nuage* germ plasm⁷ organelle (Box 1), lays the foundation to the generation of cell diversity (Knoblich 1997). The resulting plant and metazoan body plans are the integral parts over time and space of subsequent successive developmental processes (Davidson 2006). Development of a multicellular individual is mediated by the temporal and spatial regulation of expression of many thousands of genes. The core information required for gene regulatory networks to boost the phenotypic sculpturing of an adult organism is stored in the genomic apparatus consisting of the sum of chromatin embedded *cis*-regulatory modular DNA sequence elements that interact with transcription factors (Ringrose and Paro 2004). The involved cellular differentiation processes embark on embryogenesis starting from a just fertilized oocyte that now represents the zygote. The zygote is the source of all totipotent embryonic stem cells differentiating into other pluripotent stem cells from which first the embryo, and finally the terminally differentiated, mature organism arises. On a molecular scale, hundreds of thousands of minute details of physiological reactions realizing body plan development are repeated over and over again from generation to generation over vast periods of time in nearly unchanged biochemical manner. Indeed, most prominently, organisms and their descendants as a rule remain stable in their morphologies over many generations. Charles Darwin recognized this unique pattern of morphological conservation on a deep time scale. He recognized that some species like the platypus *Ornithorhynchus*, the horseshoe crab *Limulus* or the brachiopod *Lingula* survived millions of

⁶ proleptically demonstrating Virchow's 1855 statement: "*omnis cellula e cellula*"

⁷ in its contemporary meaning but not of Weismann's comprehension

years of geological time nearly unaltered in morphology, such that he coined the term *living fossil*:

"... and in fresh water we find some of the most anomalous forms now known in the world, as the Ornithorhynchus [platypus] and Lepidosiren [lungfish], which, like fossils, connect to a certain extent orders now widely separated in the natural scale. These anomalous forms may almost be called living fossils; they have endured to the present day, from having inhabited a confined area, and from having thus been exposed to less severe competition."

(Darwin 1859; [my information])

We have to invoke population biology's wisdom to realize that one of the neodarwinian conclusions was that the change of allele frequencies on a population's scale drives evolutionary change⁸. However, from the standpoint of developmental biology—and when we look at such extreme examples as *living fossils*—genetic stability rather than change seems the platform to go with. In 1892 August Weismann addressed the issue of how body plans can exist throughout geological periods unaltered in:

*"The Continuity of the Germ-Plasm as a Basis of a Theory of Heredity"*⁹

(Weismann 1892)

Weismann wondered:

*"... a cell in millions of most diversely differentiated cells that compose the body, dissociates as reproductive cell, detaches from the organism and exhibits the capability of reinstalling all peculiarities of the entire body of the new individual grown via cell division and most complex differentiation, points toward the more precise question: how does a single cell achieve to reproduce the whole with portrait-like identity?"*¹⁰

Weismann concluded that Darwin's pangenesis hypothesis¹¹ does not solve this problem (Weismann 1892, p 8). By mentioning pangenesis, however, he touched one of the most delicate matters in the history of the biology of genetic inheritance. Weismann was correct, but he couldn't know that Darwin's view on gemmulae and his deep insight (but lack of finding the right explanation) into the problems of inheritance in fact prepared the ground for the term gene—even though Darwin was not aware of Mendel's experiments. Because here it is essential to understand the crossroads between germ line, the gene, genetics and epigenetics it is necessary to explain how the term gene emerged, and to clarify the relationship between genetics and modern epigenetics:

⁸ Jim Crow, 2006, personal communication: "To me allele frequency change is the basis of evolution. There is little point in selection if it doesn't affect allele frequencies."

⁹ "Die Continuität des Keimplasmas als Grundlage einer Theorie der Vererbung".

¹⁰ translated from Weismann (1892). A year later, a 477 pages book was published in English on the same issue. Weismann A (1893) *The Germ-Plasm - A Theory of Heredity*. Charles Scribner's Sons, New York. It can be downloaded at: <http://www.esp.org/books/weismann/germ-plasm/facsimile/title3.html>

¹¹ see below

The term “Genetics” was coined by William Bateson in 1905. Yet, the term “gene” is based on ideas of Charles Darwin and Hugo de Vries. In 1878 Hugo de Vries visited Darwin, who directly stimulated de Vries to shift from physiological to evolutionary and genetic studies. The source of de Vries’ inspiration was Darwin’s speculation on heredity, i.e., the provisional hypothesis of pangenesis. According to pangenesis, hereditary characters are part of tiny cellular particles called gemmules. All cells in a multicellular organism produce gemmules during development, growth and later life. Gemmules then migrate from somatic to germ cells, where they collect to pass inherited characters to the next generation. This, actually was Darwin’s famous lamarckian statement! Hugo de Vries suggested a fundamental (and correct) modification: he abandoned Darwin’s key notion of the migration of gemmules across cell boundaries, and rechristened gemmules as “pangenes”, where the term was quickly picked up and used by Weismann as well (1893, p 235). Wilhelm Ludvig Johannsen in 1909 finally coined the term “gene” (Johannsen 1909). Only later Hermann Muller’s discovery in 1927, that genes can be altered by γ -radiation and are therefore real physical entities, and Watson’s and Crick’s discovery of the DNA structure in 1953 led to genes as the major focus of understanding the morphology of phenotypes, life histories and physiology. In 1942, Waddington defined epigenetics as “*the branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being*”.¹² Because this objective was always the driving force of biology since Darwin and before, we now understand from a traditional, historical perspective that there is a tight, inseparable connection between genetics and epigenetics on the one hand and between the germ line and passing on genes (i.e., DNA) to the next generation on the other hand.

2.3

The Emergence of Multicellular Organisms During Evolution and the Germ Line

Today our textbooks teach that multicellularity evolved several times in eukaryotes (e.g., Campbell and Reece 2005). David Penny and Richard Egel address the importance of coevolving biofilm communities in the ancestral evolution of primordial cellular life following the Last Universal Common Ancestor (LUCA) (this BOOK). After cellular life with a distinctive “haploid” genome emerged in these biofilms, individual (eukaryotic) cells of the same kind could have aggregated during phases of nutritional depletion and few cells might have become specialized in digesting other cells from the aggregate and their genomes would have combined. Haploid-diploid cycles were suggested to have evolved in an environment with an alternating degree of DNA damage (Maynard Smith and Szathmari 1997). Maynard Smith and

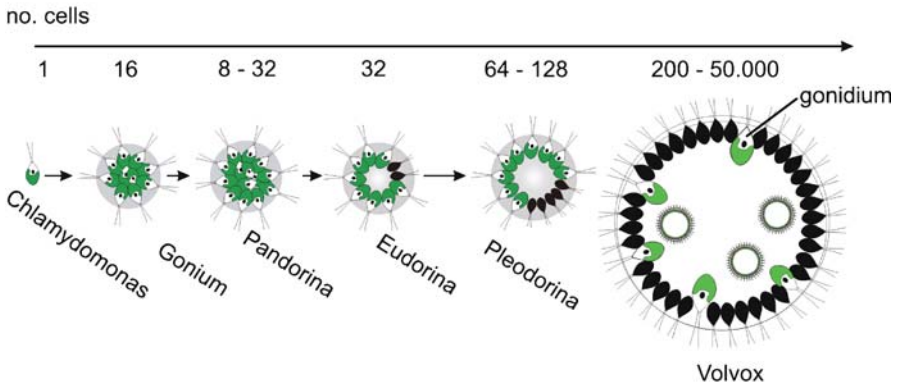
¹² The terms *phenotype* and *genotype* were first distinguished in 1911 (Johannsen 1911). See also Webconsulting FAQs and Glossary at the Epigenome Network of Excellence: <http://www.epigenome-noe.net>

Szathmáry state that the potential significance of DNA damage must have been high, because the ancient eukaryotes (being archaezoans lacking mitochondria and peroxisomes) were possibly not well adapted to coping with free radicals arising from oxygen. They could have lived in environments only if they were able to repair damage to double-strand DNA efficiently with the most ancient mechanisms of replication-related homologous recombination (J. Haber, this BOOK). If the level of oxygen in the environment alternated, archaezoans could have responded by varying their ploidy level correspondingly. Since long it is known that diploid yeast is by far more resistant to DNA damage than haploid yeast, and the latter is more resistant in G₂, after chromosome replication, than in G₁ of the cell cycle (Kunz and Haynes 1981). Most dramatically in this line of argumentation, the genome redundancy, and an ancient replicative homologous recombination mechanism termed Synthesis-Dependent Strand Annealing (SDSA) have been held responsible for the astonishing radiation resistance of the bacterium *Deinococcus radiodurans* (Zahradka et al. 2006). Even though this hypothesis of the evolution of diploidy is still indistinct, yet undefined selectional forces may have favored those quasi-cannibals in a biofilm to survive who incorporated only a single companion's genome. Such cells would have been analogous to modern diploids, where diploid genomes might have had two advantages (see below, Sect. 4.2, on a more distinct discussion about the advantages of diploidy):

1. The genetical differences of two heterogeneous haploid genomes shared in a single cell increased fitness during nutritional or other environmental stress in favor of this single cell.
2. Genetic recombination between the two genomes of the single cell would increase variation within a population of cells if the diploid genomes would separate again into multiple haploid offspring.

This partition of diploid cells into haploids would have required an accompanying invention of meiosis. The life cycle of the cellular slime mold *Dictyostelium discoideum* is a prominent model for this scenario. Thus, the evolution of meiosis likely preceded the evolution of another kind of multicellularity as we know it from metazoans. Two dimensional *Pediastrum*-like algal colonies and biofilms containing plasmodial or cellular slime mold-like communities required only low amounts of oxygen. However, three dimensional cell clusters, i.e., the ancestors of metazoans, possibly only arose when enough oxygen accumulated in the oceans, and diffusion pressure was high enough to support respiration in the inner cells of such aggregates (Hedges et al. 2004). Simultaneously, redundant (diploid) genomes were required to resist increased DNA damage caused by oxidative stress. Consistent with this assumption is recent evidence of substantial amounts of oxygen in deep sea sediments dated at 580 million years before present just to the end of the Gaskiers glaciation and shortly before macroscopic animals made their debut in Newfoundland's fossil Ediacara fauna (Canfield et al. 2007).

A.



B.

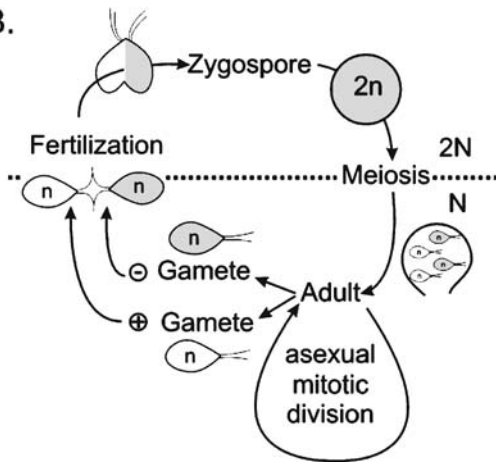


Fig. 1 Line of evolution of colony forming *Volvocinae*. **A** The line starts from a single-celled protozoan such as *Chlamydomonas*. In succession, *Gonium* and *Pandorina* represent simple colonies with 16 to 32 cells where all cells are equivalent and omnipotent. The series continues with *Eudorina* and *Pleodorina* each containing up to 128 cells. These colonial species for the first time encompass entirely somatic cells (*black*). Several of the existing *Volvox* species differentiate into somatic cells (*black*) and gametes called gonidia. Micro- and macrogametes differentiate as shown in Fig. 2 (in part based on Picket-Heaps (1975). For recent details see Kirk (2003)). **B** Sexual and asexual life cycle of *Chlamydomonas reinhardtii*. Haploid cell (n), diploid cell ($2n$), + and - gametes produce different attracting gamones and represent an early state of sex differentiation

What were these first metazoan ancestors like? Is there a good model system representing the rise of a metazoan—perhaps resembling the blastula of modern embryos in analogy to the demands of Ernst Haeckel's biogenetic law¹³ (Haeckel 1866)? Indeed, the best way to portray the evolution

¹³ biogenetic law: "Ontogeny recapitulates phylogeny"

of metazoan organisms dates back to a lecture of August Weismann held in Freiburg, in the winter 1884 (Weismann 1892). At the same time, it marks the origin of his germ-line theory. Weismann's examples included the colonial algae *Pandorina morum* and *Volvox minor*. As a useful intuition pump as outlined by Daniel Dennett (1995), we can nowadays, for operational reasons, use Weismann's example and hypothesize a line of evolution involving chlorophyll containing colonial algae (Kirk 2003) (Fig. 1A). In this line, a *Chlamydomonas* cell represents an autonomous single celled, haploid eukaryote usually living solitarily. The next evolutionary complexity is represented by three-dimensional gelatinous cell aggregates similar to present day *Gonium* and *Pandorina* colonies whose cells are all equivalent in their functional and reproductive potential. Next, *Eudorina* and *Pleodorina* represent the first hollow-sphere colonies in which up to half of the cells lose their reproductive capabilities and are solely somatic (black cells in Fig. 1A). *Volvox* finally represents a chlorophyll containing hollow colony consisting of up to ~50 000 cells where most cells are differentiated into specialized somatic cells attached to a ball of mucilage.

Indeed, August Weismann took advantage of these model examples as follows (Weismann 1893, p 213): "*In my opinion, germ-cells were sharply distinguished from somatic cells on their first appearance in phylogeny, and this distinction has since persisted. ... I know of no more convincing proof of my view than that which is furnished by the Volvocinae. These organisms consist of communities of cells which may or may not exhibit a division of labor, and in which a contrast between the somatic and germ-cells may or may not exist. In Pandorina all the cells of the colony are similar to one another, and each performs all the vital functions. In Volvox the cells are differentiated: some of them have the function of maintaining the individual, and others that of preserving the species: that is to say, they are differentiated into somatic cells and germ-cells. The heteroplastid genus Volvox must have arisen phyletically from a homoplastic form: but we can hardly imagine that there can be many intermediate stages between these two, for at present day the two kinds of cells in Volvox hardly differ as much as do the somatic- and germ-cells in the case of higher organisms. The somatic cells have nevertheless entirely lost the capacity of reproducing the entire organism.*"

2.4

Amphimixis and Meiosis

Weismann also recognized the need for reducing the quantity of the hereditary substances (called germ plasm in his terminology) and chromosomes after two individuals became united (Weismann 1893, p 235): *The Necessity of a Halving of the Germ-Plasm. By the process of amphimixis*¹⁴ *the hered-*

¹⁴ Amphimixis is the union of gametes in sexual reproduction.

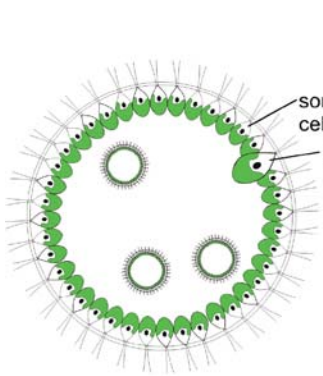
itary substances of two individuals become united into one substance in the offspring. If the process is repeated in every generation, a doubling of these individually different hereditary substances must take place each time, and the mass of germ-plasm and the number of idants [=chromosomes] must likewise be doubled. As a matter of fact this cannot and does not occur, for in every species the number of idants remains the same throughout all generations. The unlimited increase of the germ-plasm must therefore be prevented in some way or other ... This actually occurs before the germ-cells unite in the process of 'reducing division' of the nuclear matter of the germ-cells.

How does Weismann's insight match the evolutionary progression line of the *volvocine* green algae (Fig. 1A) with how we comprehend germ line, meiotic division and sexual reproduction today? *Chlamydomonas* is a single-celled haploid eukaryote with a chloroplast, a stigma and two flagella. Figure 1B shows its life cycle. It reproduces asexually (by mitosis) when conditions are favorable. Sexual reproduction occurs when conditions become unfavorable. The diploid zygote forms a thick-walled zygospore that is resistant to environmental extremes and divides by meiosis when environmental conditions become favorable. Most species of *Chlamydomonas* are *isogamous*¹⁵, some are *oogamous*¹⁶. The isogametes (+ and -) of *Chlamydomonas reinhardtii* produce gamones which interact with surface components responsible for sexual flagellar contact. Other *Chlamydomonas* species produce anisogametes. Several authors have suggested reasons for why gamete dimorphism may have evolved (Maynard Smith and Szathmary 1997). One model predicts that Brownian motion alone is enough to favor the evolution of gamete dimorphism by increasing the rate of encounters between the two types of gametes (Schuster and Sigmund 1982). Further it was suggested that anisogamy would evolve from isogamy by disruptive selection if larger zygotes were selectively favored over smaller zygotes (Maynard Smith 1978). Other authors suggest that anisogamy will evolve if one genotype is favored because the large size of its gametes enhances survival of the offspring, and another is favored because it can make many gametes (Charlesworth 1878; Hoekstra 1987). Similar selective forces on a higher level may have been responsible for the evolution of multicellular male and female organisms. Thus, the same principles may have become transferred to the level of cell communities upon which selection acted as individual units of selection. Such individualized cell-collectives represent an ancestral state of metazoans and can be found in *volvox*-like cell collectives. *Volvox* is a colonial green algae. It is the most simple, quasi-living fossil multicellular organism that produces a carcass after offspring colonies have been generated and released. Its cells are arranged in a hollow, gelatinous, blastula-like sphere each with a chloro-

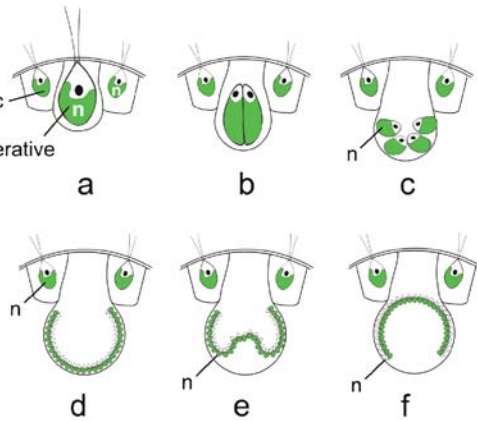
¹⁵ If the gametes have the same size and form they are called *isogametes*.

¹⁶ *Oogamous* gametes differ in size and in shape, e.g., small motile male gamete (sperm) and a large immobile female gamete (egg). On the other hand, *anisogamous* gametes just differ in size but not in shape.

A.



B. *Volvox* Asexual Reproduction



C. *Volvox* Sexual Reproduction

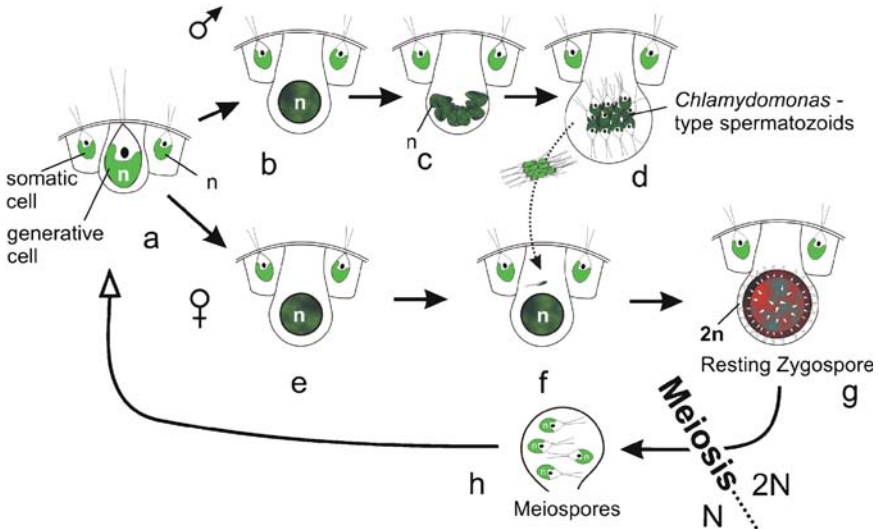


Fig. 2 *Volvox*, vegetative and sexual reproduction. **A** *Volvox* colony with somatic and generative cells. **B** *Volvox*, asexual reproduction. (a–f) Formation of daughter individuals. All cells are (n) haploid. **C** *Volvox*, sexual reproduction cycle. (a–d) Male germ line, development of spermatozoid. (a,e,f) Formation of oocyte. (f) fertilization, (g) diploid zygote and zygospore formation, (g–h) meiosis and release of haploid megaspores (in part based on Essex (1976))

plast, a stigma and two flagella directed to the outside. The monoecious¹⁷ *Volvox globator* encompasses 1500 to 20000 cells. The dioecious¹⁸ *Volvox*

¹⁷ having male and female sex organs in the same individual

¹⁸ having male reproductive organs in one colony and female in another

aureus contains 200–3200 cells. These colonies reproduce both vegetatively as well as sexually (Fig. 2). “*The germ-plasm of Volvox must therefore contain spermatogenetic and oogenetic determinants besides those for the ciliated somatic cells, only one or the other of which, however, becomes active, and impresses the male or female character on the germ cell.*” (Weismann 1893).

Similar to *Chlamydomonas*, *Volvox* divides asexually through mitotic divisions to produce daughter colonies segregated to the hollow interior of the sphere (Fig. 2A). The difference to *Chlamydomonas* is that somatic cells are sterile and do not reproduce. In the line of progress the first colonies that produce somatic cells are *Eudorina* and *Pleodorina* (Fig. 1A). In *Volvox*, at first, about 16 totipotent, haploid, generative cells called gonidia are set aside from the majority of somatic cells (Fig. 2Ba). These divide mitotically (i.e., asexually) and produce a hollow mucilage-filled ball bounded by a single layer of cells (Fig. 2Bb–d). This very much resembles the blastula of an early metazoan embryo. Even a gastrulation-like process occurs, which turns the cell-layer completely inside out (Fig. 2Be–f). A *Volvox* colony further sets apart a couple of cells which, in contrast to somatic cells, retain a sexual-reproductive potential—these cells are sexual germ-line cells *sensu stricto* (Fig. 2C). The sexual germ-line cells of *Volvox* develop into two different kinds of heterogamous germ cells (male and female), i.e., the smaller male micro-gametes (Fig. 2Cb–d) and the larger female macro-gametes (Fig. 2Ce–g). Dioecious male colonies can produce a gamone that induces other *Volvox* colonies in the vicinity to become sexually active. As a unit, the sperm packets are released into the aquatic environment and actively swim away in search of *Volvox* eggs (Fig. 2Cd,f). After successful fertilization, a resting zygospore forms that incorporates a thick, spiny cell wall that is often red in color, and that can survive winter environment. Within the resting spore, meiosis occurs and each resulting meiospore evolves into a new colony (Fig. 2Ca,g–h) (Esser 1976; Kirk 2005).

2.5

On the Value of the *Volvocinae* as a Line of Evolution Towards Multicellularity

The linear evolution drawn in Fig. 1A launches with *Chlamydomonas*, continues with *Gonium*, *Pandorina*, *Eudorina*, *Pleodorina* and concludes with *Volvox*. These green flagellates have been estimated to have shared a common (single cell?) ancestor only 50 My ago—thus well after dinosaurs became extinct and around the time Baltic amber formed and the horse-ancestors roamed at Messel, Germany (Kirk 1998; Rausch et al. 1989). If these calculations are true, *Volvox* is no true *living fossil* that survived as a blastula-like metazoan ever since the origin of bilateral metazoans at around 580 My ago (Hou et al. 2004). It rather may represent some sort of belated parallel evolution¹⁹ from a single-

¹⁹ parallel evolution: independent evolution of similar traits, starting from a similar ancestral condition

celled eukaryote some 500 My later. Nevertheless, the *Volvocinae* do represent a didactic model for the evolution of multicellularity and the germ line. As used by Weismann, this model still well symbolizes the emergence of the germ line with meiosis reducing the doubled genetic informational content into a haploid genome. Further, it resembles the evolution of gamete dimorphism to maintain sex in males and females, and is an instructive model for the first metazoan capable of dying and producing a true carcass.

2.6

Chromatin Diminution:

The First Hints in History Towards Germ-Line/Soma Segregation

Nowadays it is evident that somatic cells often possess increased or decreased amounts of DNA relative to the diploid germ-line cells. For example, the human erythrocytes lack a cell nucleus and nuclear DNA. The chorion genes of silk moth and *Drosophila* are amplified in specific tissues (Iatrou et al. 1984; Levine and Spradling 1985; Mitsiatis and Kafatos 1985). The salivary glands of *Drosophila* larvae produce thousands of copies of their chromosomes. Similar polytene chromosomes also occur in suspensor cells of plant embryos (*Phaseolus vulgaris*). Historically, the legacy of these cognitions belongs to the core of Weismann's essential insights into embryonic animal development. Weismann assumed that all the chromosomes which remain intact in successive generations carry all the hereditary information needed to make a whole individual. Every single chromosome would be responsible to determine a particular part of an adult body. Further, each chromosome was assumed to be composed of smaller subunits termed *biophores*²⁰ (Weismann 1893). The essential point was his realization that *biophores* were distributed unequally to the somatic daughter cells during embryonic development, explaining cell differentiation by somatic gene segregation. (Nowadays, epigenetics has taken up this heritage in only a slightly modified yet hotly debated fashion (Chong et al. 2007; Horstehmke 2007; Suter and Martin 2007), now mainly dealing with chemical modifications of chromatin-proteins, prions and nucleic acids, investigating their medical relevance and the influence on somatic development). The causal link between differential distribution of genes in the various body parts was set by Theodor Boveri who described in 1887 chromatin diminution in the nematode *Parascaris equorum* (Boveri 1887). Today, *Caenorhabditis elegans* serves as a prime model organism where the entire developmental cell lineage with the fixed cell number of cells from the zygote to the adult is known (Soulston 2002). Weismann used the cell lineage of *Ascaris nigrovenosa*, to sketch the first diagram of the germ line in relation to cell lineage development (Weismann 1893, p 196). Figure 3 depicts the lineage of *Parascaris equorum* with germ-line/soma segregation in one

²⁰ *Biophores* are genes, defined by Weismann as “bearers of vitality, the smallest vital units”.

- ◀ **Fig. 3** Deep time and the segregation of germ line and somatic cells in *Parascaris equorum*. **A** Chromatin diminution in early cleavage divisions. (a) Anaphase of 1st cleavage division. (b) Anaphase of 2nd cleavage division. Chromatin diminution as indicated by lost chromosomal fragments. It occurs in the top S_1 cell but not in the lower P_1 cell. (c) Four-cell stage after completion of the 2nd cell division. The cells S_{1a} , S_{1b} , and S_2 are the founders of somatic cells. P_2 is the presumptive primordial germ cell (based on Tobler (1986)). **B** Serial phases of germ cell lineage and somatic cell differentiation. Each cuboid represents an individual of one generation. Successive ancestor generations wane in deep time to indicate the potential immortality of the germ line (red)

stage P_1 again distributes the two chromosomes normally to the two daughter cells P_2 and S_2 . The S_1 cell, however, loses chromosome fragments and gives rise to cells S_{1a} and S_{1b} which have undergone chromatin diminution (Fig. 3Ac). Further, cells S_2 thru S_4 also lose totipotency by getting rid of chromatin and become pluripotent somatic stem cells (Fig. 3B). All somatic cells have lost their capacity to be part of the germ line while the germ line stem cells P_0 through P_3 represent the presumptive primordial germ cells (pPGC). The pPGCs are the forerunners of primordial germ cells (PGCs), but they still give rise to both the germ line and somatic cells. P_4 is the first PGC. Like monoecious plants *Parascaris* is a hermaphrodite segregating male and female gametes in the same individual. Nematodes are not entirely hermaphroditic. For example, *Caenorhabditis elegans* is a hermaphrodite with two ovaries, oviducts, spermatheca, and a single uterus. But 0.05% of a worm population are male only. Hermaphrodite *C. elegans* have a matched pair of sex chromosomes (XX); the rare males have only one sex chromosome (X0). This brings up the question of how diverse germ-line/soma segregation processes can be in the animal and plant kingdoms.

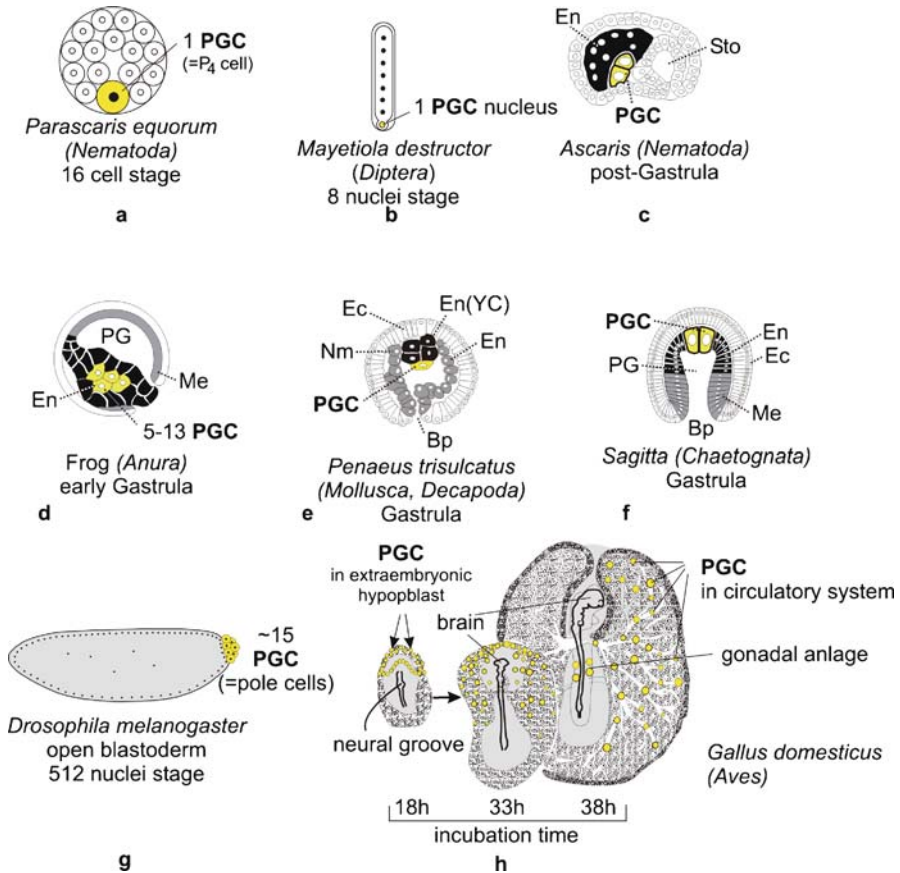
2.7

Biodiversity, Germ-Line Versus Soma Segregation and Preformation Versus Epigenesis

The most significant key characteristic of the germ line missed so frequently in common definitions (see dictionary entries above) is the transmission of the entire inheritable, genetic and epigenetic information from generation to generation. Developmental biologists still debate two distinct modes of germ-line segregation, i.e., preformation²¹ and epigenesis²² (Extavour and Akam 2003). However, the germ line always passes on its genetic information uninterrupted from grandmother and grandfather to the grandchildren no matter of what is in-between, or when and how germ cell differentiation occurs relative to the de-

²¹ **Preformation:** germ cells can be easily identified in early embryogenesis, when their differentiation is assured by localization of maternally inherited determinants before, or immediately following, fertilization.

²² **Epigenesis:** germ cells are not observed until later in development, and “arise” as a result of inductive signals from surrounding tissues.



velopment of somatic tissues. The zygote is the ultimate totipotent germ-line stem cell but it is also the first pPGC. In different species the PGCs segregate from the pPGCs, and the gonial germ cells segregate from the PGCs at various time points during cell lineage development. As shown for *Drosophila*, gonial stem cells can be lost from a stem cell niche²³ but regenerate through dedifferentiation from primary gonial cells (Wallenfang et al. 2006). Germ cells can develop from one single PGC such as in *Parascaris equorum* and in *Cyclops* or from even a single nucleus as in the dipteran insect *Mayetiola destructor* (Figs. 3B and 4a) (Extavour and Akam 2003; Tobler 1986). In most other metazoans such as *Xenopus laevis* and *Drosophila melanogaster*, PGCs can be traced back to multiple cells or nuclei in the early embryo (Fig. 4d,g).

²³ Stem cells retain the capability to divide and to develop into many types of adult cells. A stem cell niche represents a specialized cellular environment that provides stem cells with the support needed for self-renewal. A germ-line stem cell niche consists of germ-line stem cells and supporting somatic cells.

- ◀ **Fig. 4** Diversity of numbers and localities of PGCs in the embryos of various metazoans. Germ-line cells (*yellow*). **a** Very early determination of germ-cell fate in a nematode, and **b** in the dipteran gall midge *Mayetiola*. **c** “Later” determination of germ cell fate in another nematode, **d** a frog, **e** a mollusc, **f** an arrow worm, and **g** the fruitfly *Drosophila*. This dipteran insect serves as a general role model for germ plasm determination, and many of the key genes have been identified by genetic maternal effect screens. Early embryonic nuclei divide in the syncytial (plasmodial) ooplasm. Few nuclei migrate and become embedded within the pole plasm. These become individualized as pole cells that represent the first differentiated germ line cells of the new generation (see Box 1 for related current concepts on germ plasm). **h** Early on, chicken PGCs were thought to originate from the extraembryonic hypoblast (*arrows*) (Fioroni 1987; Swift 1914) until 1981. Then, experiments using chick-quail chimaeras made before primitive streak formation showed they were of epiblastic origin. Isolation of a chicken *vasa* homolog has now made it possible to trace pPGCs back to the cleavage stage embryo. Chicken Vasa protein forms part of the *nuage* in chick oocytes, and localizes to cleavage furrows until stage IV, when six to eight cells of the ~ 300 cell embryo contain Vasa and likely represent PGCs (Tsunekawa et al. 2000). This suggests that preformation is the mechanism for germ-cell specification in chickens (Extavour and Akam 2003). Later in development, PGCs migrate via the circulatory system into the presumptive gonads (gonadal anlage). *Hypoblast*: Old term for the inner germ layer of *Sauropoda*; the origin of the entoderm. *Epiblast*: The embryonic layer of vertebrate embryos from which the embryo arises during gastrulation; origin of all germ layers of the embryo. En, entoderm; STD, stomodaeum; PG, primary gut; Me, mesoderm; Ec, ectoderm; Bp, blastoporus; CY, yolk cell; Nm, nauplius mesoderm

Similar to plant meristems, the future germ cells of animals are thought not to be always linear descendants from a single, specific, stem cell or nucleus. For example, the highly differentiated choanocytes of the sponges *Aplysilla* and *Clathrina* can transform into germ cells (Fioroni 1987; Siewing 1969) (Fig. 5). Analogous to *Volvox* colonies (Fig. 1), the choanocytes of sponges, solitary choanoflagellates and choanoflagellate colonies such as *Proterospongia haeckli* represent a progression line of metazoan evolution, although this line is likely to be much more ancient than the *Volvocinae*. In this light, the dedifferentiation of sponge choanocytes into phagocytosing oocytes appears similar to the gonidia of *Volvox* and therefore may in fact be no “dedifferentiation” sensu stricto after all (Fig. 5). This often blurs the germ-line concept (Shostak 2006) and prompts authors to debate whether species *with no apparent germ line* (Tobler 1986, pp 10–11) segregate only very late so that an actual germ line goes undetected. The argumentation then denies that germ-line-soma differentiation precedes all somatic differentiation. This, so the argument, is clearly not the case in a wide range of invertebrates and of course in plants, where germ cells can arise from “*undifferentiated pluripotent reserve cells*” (Tobler 1986). Part of this recurring problem led Charles Darwin to introduce *gemmules* in his hypothesis of pangenesis (see above). Here, “*pangenetic*” information engraved into a somatic cell is potentially in state to be transferred into the next generation. However, in the light of just passing on identical information of inheritance (i.e., DNA and epigenetic information) in a mathematical sense from

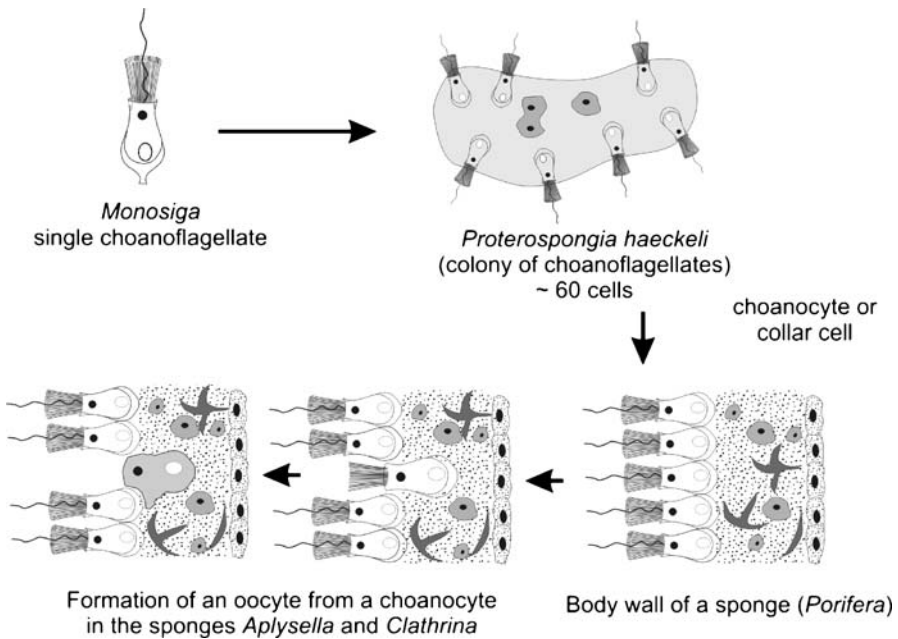


Fig. 5 Progression line of evolution from single-cell choanoflagellates to sponges

generation to generation, and to carry on the integrity of a species as a biological entity, the debate of whether the germ line encompasses cells from somatic origin or not appears futile. More important is the question of whether the germ line is monophyletic and ancestral to metazoa if not even including plants as well. Germ cell specification has been recognized as an ancestral feature of metazoans (Extavour and Akam 2003) and it is part of the same road towards understanding the role of the germ line in evolution.

One strong argument of the putative monophyletic origin of the germ line is the ubiquitous conservation of *vasa* gene-encoded RNA helicase and its orthologs in metazoans (Schüpbach and Wieschaus 1987). It is detected in germ-line cells wide spread throughout the life cycles of metazoans (Ikenishi 1998; Mochizuki et al. 2001). Another germ-line marker is *nanos*, a gene encoding a CCHC Zn-finger protein involved in translational and transcriptional repression in most tested metazoans. Further, the *boule* gene encoding an RNP-type RNA binding protein with DAZ repeats which is involved in meiosis and PGC differentiation (Extavour and Akam 2003). Supportive of the rationale that the germ line is monophyletic however, are not only these gene germ cell markers universal to metazoans. There must be more: Across phyla PGCs contain in their cytoplasm some kind of aggregate of electron-dense, basophilic bodies. These aggregates are termed differently in various organisms, i.e., *dense bodies*, *nuage*, *mitochondrial clouds*, *chromatoid bodies*, *yolk nuclei* or *Balbani bodies*. These features are observed at some stage of all an-

imal phyla examined by electron microscopy (Eddy 1975; Extavour and Akam 2003; see also Box 1).

Thus, on the one hand, the specification of germ cells by epigenesis and preformation is part of the germ-line concept and helps to define the germ line as an evolutionary conserved hereditary entity. On the other hand, whether the genetic/epigenetic information defining an organism will be passed on to the next generation fully intact only depends on three parameters:

1. mutations fixed in the genome of the novel zygote,
2. alternative molecular epigenetic modifications deposited within the new zygote, and
3. chromosome assortment and rearrangements created through meiotic segregation and recombination.

To what extent partially differentiated tissue cells can become dedifferentiated is certainly relevant to know. From a medical point of view, especially concerning stem cell applications and research, the specific reprogramming pathways are utmost important to understand. However, how the reprogramming of epigenetic and epigenomic tags, back to a developmental, basic zero state works and is fed back into the germ line remains of lesser importance or is even irrelevant in an evolutionary context (Fig. 4). The crux of the matter always remains what identical information of inheritance properly passes on from generation to generation. Meiotic recombination, mending of DNA damage, and the activities of transposable elements are the main control elements of germ-line genomes. This has been termed the *quantum dimension of biological phenomena*, where the difference of cleavage of a particular phosphodiester bond resolving a Holliday junction in the germ line decides whether the emerging organism is alive or dead (Falaschi 2007, see below).

2.8

Linking Weismann's to Current Views on the Germ Plasm

When Weismann first conceived of his farsighted ideas as to the continuity of the “germ plasm” he based this on chromatin diminution (Sect. 2.6) and on his belief that chromosomes were stainable and thus real physical entities²⁴. However, the precise distinction between “chromosomes” and germ plasm was blurred. It was well before a common understanding had been reached about the inner workings of a living cell. Everything inside a rigid cell wall was referred to as the protoplasm. A functional distinction between the nucleoplasm and the surrounding cytoplasm was only just arising,

²⁴ Weismann's coeval colleague Walther Flemming discovered mitosis in 1882. He used aniline dyes staining chromatin and chromosomes.

but genes as instructive repositories for the expression of particular catalytic activities were not yet known. Indeed, before Muller's 1926 X-ray experiments on *Drosophila*, genes could not be identified as real physical entities (see Lankenau 2007). In fact, Weismann was among the earliest influential protagonists for a nuclear localization of the hereditary potential from one generation to the next, and thus he correctly linked his "germ plasm" to the nuclear chromosome activities prevailing in germ cells and their progenitors, as contrasted with the various diminished versions of "idioplasm" in differentiated soma cells. Later on, when genes had become firmly established and their central role in controlling cellular metabolism had been resolved, the germ plasm *sensu* Weismann lost its former standing as an influential term—being superseded by genes and genomes. On the other hand, the accumulating experimental evidence regarding strictly localized cytoplasmic cues for germ cell determination demanded a distinctive term for this phenomenon alone. This led to a revival of the *germ plasm* term in new guises. On a heuristic point of view this hardly is an infringement on a copyright that had long since expired. Rather, it should bring even wider acclaim to Weismann's ingenious insights by his modern respondents. For a link to the current concepts joining the definition of life, the germ line and germ plasm see Box 1.

Box 1 Germ-Line Signatures and Cytoplasmic Means

I. Combining the definition of life with the germ-line concept:

- a) *It is to define as alive any entities that have the properties of multiplication, variation and heredity. These entities are called "replicators" (Dawkins 1989; Muller 1966).*
- b) *Metabolism supplies the monomers from which replicators (i.e., (epi)genomes and chromatin) are made.*
- c) *Replicators alter the kinds of chemical reactions occurring in metabolism.*
- d) *Only if b) and c) are true can natural selection—acting on replicators— influence the evolution of metabolism (Maynard Smith and Szathmary 1997).*
- e) *First in evolution, Weismann's hierarchical selection established single-cellular organisms and later multicellular metazoans (Table 1).*
- f) *Unicellular organisms are potentially immortal.*
- g) *The metazoan germ line is potentially immortal on a higher selectional level.*
- h) *Mortal somatic cells segregate from the germ line and not vice versa.*
- i) *The nuage organelle is the extended arm of the germ-line genome, i.e., the key executive institution implementing the genetic information encoded within the (epi)genome.*

II. Current conviction on the germ-line concept is in part based on genetic theory
Classical genetics, cytogenetics and population genetics established the theoretical framework as to the existence of the transgenerational germ line—originally and unwittingly as a rational concept by reasoning rather than a physical entity.

Box 1 (continued)**III. The (epi)genomic platform of germ-line maintenance**

The evolution of potential immortality of the germ line required the establishment of an enduring genome-stability-platform represented by contemporary metazoan (epi)genomic chromatin metabolism. This platform must be able to control and to balance gene expression and genome dynamics as represented by the relative weighting of replication, DNA-repair, meiotic recombination and DNA modifying activity of transposable elements. The above made statement (1d) only operates when chromatin metabolism in germ-line cells was selected such that mutation levels became stabilized in a well-orchestrated manner. Almost nothing is known about germ-line-specific DNA metabolism and urgently awaits future attention.

IV. The *nuage* organelle—a key signature of the germ line.

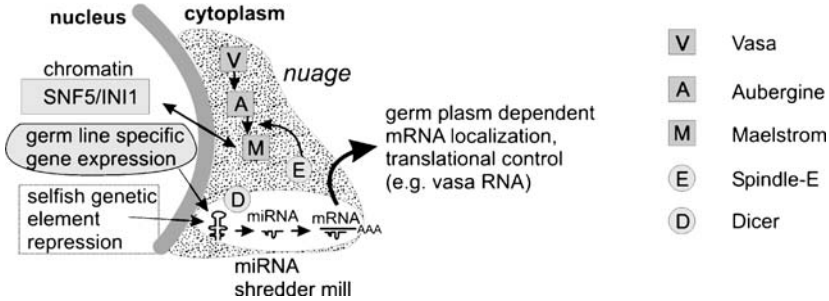
In 1957, André and Rouiller first coined the term “*nuage*” (i.e., cloud in French) (André and Rouiller 1957). Its amorphous and fibrous structure occurred in drawings as early as in 1933 (Risley 1933). Today, it is accepted to represent a characteristic, electron dense germ plasm organelle encapsulating the cytoplasmic face of the nuclear envelop of cells destined to the germ-line fate. The same granular material is known as *dense bodies*, *mitochondrial clouds*, *yolk nuclei*, *Balbani bodies* or *perinuclear P granules* in *Caenorhabditis elegans*, *germinal granules* in *Xenopus laevis* and *chromatoid body* in mouse (for review see Eddy (1975); Extavour and Akam (2003); see also Fig. 4g: *Drosophila* pole cells becoming determined in pole plasm). Molecularly, the *nuage* is a tightly interwoven network of differentially localized RNA-binding proteins, which in turn localize specific mRNA species for differential storage, asymmetric segregation (as needed for asymmetric cell division), differential splicing and/or translational control—perhaps even providing a shredder mill for miRNA molecules that could feed back on the nuclei for suppressive control at a grander scale. The *nuage* appears to be ancestral and universally conserved in the germ lines of all metazoan phyla. Signified by *nuage* specific protein components such as Vasa, Nanos, Aubergine, Maelstrom and many others, recent work suggests a common origin of germ cells and of somatic stem cells in the most basal branches of the Metazoa such as *Porifera*, *Cnidaria*, *Placnaria*, and *Annelida* (Extavour and Akam 2003; Rebscher et al. 2007). Roughly, two functions have been assigned to the *nuage*:

1. Regulation of germ-line-specific gene expression circuits and related phenomena (recent examples of a growing body of literature are: (Chuma et al. 2006; Hosokawa et al. 2007; Johnstone et al. 2005; Knaut et al. 2000; Shirae-Kurabayashi et al. 2006; Snee and MacDonald 2003)).
2. Repression of the proliferation of selfish genetic elements capable of mutationally destabilizing germ-line genomes (Brennecke et al. 2007; Lim and Kai 2007; O'Donnell and Boeke 2007).

Nuage components are assumed to be assembled in a hierarchical relationship. The DEAD-box RNA helicase Vasa is thought to function as a recruiting “platform” that lies in the perinuclear region and recruits subsequent *nuage* components such as Aubergine, Piwi, Tudor, Spindle-E or Krimper, just to mention a few (Findley et al. 2003; Lim and Kai 2007). Further, Maelstrom associates with the chromatin remodelling complex SNF5/INI1, indicating that *nuage* may regulate the chromatin stage to repress unfavorable gene expression in germ-line cells (Costa et al. 2006).

Box 1 (continued)

Thus, the highly conserved nature of the *nuage* throughout evolution strongly implies its importance and essentiality in the germ line. See Sect. 2.8 for a link to Weismann's germ plasm.

**3****The *Allmacht*²⁵ of Selection****3.1****Different Levels of Selection—Kin Selection**

The hierarchical theory of selection recognizes many kinds of evolutionary individuals, banded together in a rising series of increasingly greater inclusion, one within the next—genes in cells, cells in organisms, organisms in demes, demes in species, species in clades. The focal unit of each level is an individual, and we may choose to direct our evolutionary attention to any of these levels. Once we designate a focal level as primary for a particular study, then the unit of that level—the gene, or the organism, or the species, etc.—becomes our relevant or focal individual, and its constituent units become parts, while the next higher unit becomes its collectivity.

(Gould 2002, p 674)

In his classical work *Animal Species and Evolution*, Ernst Mayr states in his introduction that even today there are still zoologists to whom the term “Evolution” means little else but making decisions about homologies, common ancestors, and phylogenetic trees. However, by far most evolutionary biologists focus their interest on studies dealing with the causes and the mechanisms of evolutionary change and try to establish the relative role and importance of different factors (Mayr 1963). His conclusion is still accurate

²⁵ omnipotence and/or almightiness

today and this book is meant to lead into this direction. If it turns out to be correct that the PGCs, as a specialized cell type, may well be homologous across all metazoa, by the criterion that they have retained an ancestral suite of molecular characteristics that define the germ cell lineage (Extavour and Akam 2003), then the metazoan germ line itself being defined as the pipeline transporting the substances of inheritance beyond generations might be monophyletic as well. For this reason it is not only legitimate but forcefully mandatory to ask: how and why did the germ line and maintenance of sex in metazoans arise? This question includes a key insight, and it is not just a ludicrous phrase trying to shield a weak argument (Schön et al., this BOOK). The evolution of multicellularity and the origin of a germ line obviously go hand in hand. In this respect, August Weismann himself made a tremendous contribution almost forgotten by today's contemporaries. His major contribution in fact was the recognition of hierarchies of selection. Weismann realized a level of "subcellular selection" (originally meaning between cells but not within cells) where germ cells competed among each other and with somatic cells for existence, and he called it "germinal selection". Stephen Jay Gould worked out these cutting-edge insights of Weismann (e.g., Weismann's remarks on unfertilized bee offspring developing into male bees (Weismann 1892)) which actually, in the 1960s, culminated in Hamilton's modern theory of kin selection (see below). In addition, Gould discovered a hidden truth about Charles Darwin's beliefs about hierarchies of selection (Gould 2002). Let Stephen Gould speak for himself (Gould 2002, p 63, pp 197–208): "*In the first generation of Darwinian debate, August Weismann, ..., the only biologist (besides Darwin) who fully grasped the logic and implications of selection, wrestled with levels of selection throughout his career. ... He began by trying to refute Lamarckian inheritance by advocating the Allmacht²⁵ ... of natural selection. He first attributed the degeneration of previously useful structures to what he called 'panmixia' (not the modern meaning²⁶ of the term, but the effect of recombination, in sexual reproduction, between adaptive elements and inceptive elements no longer subject to negative selection); then realized that this process could not explain complete elimination,²⁷ thus leading him to propose a lower level of subcellular selection, potentially acting in opposition to organismal selection, and called 'germinal selection'; and finally recognized that if levels of selection existed below the organismal, then the same logic implies the existence and potency of supraorganismal levels as well. ... Darwin realized that he needed to invoke species selection Wallace²⁸ simply didn't grasp the concept of levels at all. ... Darwin, by contrast, completely understood the problem of levels ...*".

²⁶ modern meaning of panmixia: random mating within a breeding population

²⁷ Degeneration followed by elimination is the loss of a character when selection forces are lacking. For example, loss of eyes in cave animals.

²⁸ Alfred Russell Wallace (1823–1913) independently of Charles Darwin proposed a theory of natural selection.

The finding that Charles Darwin realized the need of group selection is surprising and remarkable. Traditionally, Darwin's following statement stands as doctrine of contemporary thought: "*Hence, as many more individuals are produced than can possibly survive, there must in every case be a struggle for existence, either one individual with another of the same species, or with the individuals of distinct species, or with the physical conditions of life.*"

Therefore, we think of Darwin being committed to selection between individuals (or organisms) within a population or individuals between populations only. To the contrary, Gould finds that Darwin used organismic evolution mainly for tactical reasons to be more efficient in convincing contemporaries about the fact of evolution and of its mechanism, natural selection. Gould cites Ruse who writes (Ruse 1980, p 620): "*By the end of the decade (the 1860s) ... there was nothing implicit about Darwin's commitment to individual selection. He had looked long and hard at group selection and rejected it.*" Then Gould continues in his analysis: "*In my researches ... [on Darwin] ... (Darwin 1975, e.g., p 239)²⁹ I made a discovery that strongly supports this view of Darwin's attitude towards supraorganismal selection. I found that the traditional sources (Ruse, Kottler and others) did not identify Darwin's major, explicit struggle to contain an apparent need for higher-level selection, and to assert exclusivity for the organismal mode. He fought a far more important battle with himself on an issue well beyond particular problems raised by single taxa (sterility of worker castes or human morality): the explanation of the principle that he ranked second only to natural selection itself as a component of evolutionary theory—the 'principle of divergence'.*"

Weismann, on the other hand—via his university lectures—was more accessible to the public, and he applied the problem of the reduction and loss of organs with the absence of negative selection for a theory of germinal selection. Gould elaborates on this (Gould 2002, pp 197–208): "*Weismann's ... attempts to explain degeneration by panmixia. ... Panmixia is a genuine, but weak force; it can reduce the average value of an organ to a state somewhat below its former functional size. But panmixia cannot solve the central question of degeneration: what propels a useless organ ... into history's dumpster?*"

Weismann admitted his failure (1896, p 22) (Weismann 1896). ... *He needed ... another kind of hypothesis to preserve the Allmacht of selection against resurgent Lamarckism. He had tried the mechanics of inheritance as expressed in the doctrine of panmixia; now he would expand the domain of selection itself. He would depart from Darwin's [as Weismann reflected on Darwin] distinctive focus on struggle among organisms, and attempt to identify a source of directional variation in an analogous competition among determinants of heredity within germ cells —a 'germinal selection'. Weismann devised a truly*

²⁹ Charles Darwin's unabridged forerunner-manuscript drafts to the *Origin of Species* were written between 1856 and 1858, and finally published in 1975 by R. C. Stauffer.

ingenious argument: if natural selection can produce trends in the morphology of phenotypes, then an intracellular, germinal selection might yield directionality in the variation presented to conventional selection upon organisms. If the determinants of a useless organ predictably lose in an intracellular struggle for existence, then a trend to complete elimination—...—might still be attributed to selection. ... Germinal selection applied to replicating objects at a subcellular scale rather than to entire organisms.”

Thus, the superimposition of multicellularity and germinal selection appears to be the cause of the origin of germ line differentiation programs. Beyond that, as organismic selection had been worked out so brilliantly in Darwin's *Origin of Species* and in the *Descent of Man and Selection in Relation to Sex*, extrapolation of germinal selection would clear the way for higher levels of selection, i.e., group selection. How this translates into modern understanding of the origin of the germ line together with multicellularity becomes evident from a very perceptive definition of Bell (1997). He characterizes the evolution of development of multicellular organisms as tightly coupled to the evolution of the germ line in the following way (p 522): “*The most familiar and highly organized clonal aggregations are the bodies of multicellular organisms. The development of any but the simplest multicellular creature involves the death or sterilization of many of the cells involved. The germ line may be segregated early in development (as in Volvox, or vertebrates), or it may be continually recruited from a population of stem cells (as in polyps and plants), but in either case, a cell that has become differentiated for a particular somatic function very rarely gives rise to germ cells.*”

Bell testifies unpublished experimental evidence for the selective advantage of somatic cells in *Volvox* (Bell 1997). As shown in Fig. 2, *Volvox* is a hollow sphere of about 10 000 cells, the great majority of which are small somatic cells resembling *Chlamydomonas* (Fig. 1); a dozen or so much larger germ cells (gonidia) are set aside early in development by an unequal division. The germ cells develop into new colonies that are eventually released from the parental colony, leaving it as a hulk of somatic cells that soon die. Koufoparou and Bell macerated colonies to isolate germ cells, which are capable of growing as unicells in nutrient medium, in the absence of a soma. The growth rate of these isolated germ cells, however, was less than that of the intact colonies. The rate of proliferation of the colonial clone as a whole is thus increased when most of its members are unable to reproduce, but serve instead to accelerate the reproduction of a small set of clone mates (Bell 1997, p 523). In nature, the biomass of species investing in soma-cells increases proportionate with increasing eutrophication of ponds relative to the biomass of species lacking separate soma cells. This suggests a “source and sink” model for the advantage of multicellular organisms with soma/germ-line differentiation (Koufoparou and Bell 1993).

While Weismann's (*germinal selection*) and Darwin's levels of selection serve as an explanation of germ cells separating from somatic cells in analogy

to the *Chlamydomonas*–*Volvox* line of evolution (Fig. 1) selection further can drive the evolution of yet higher levels of colonial assemblies. For example, the zooids of some colonial hydrozoans and bryozoans develop as purely somatic creatures, serving the reproductive zooids of the colony. A typical *Hydractinia* colony is composed of a network of gastro vascular canals, termed stolons, from which polyps (i.e., zooids) arise (Frank et al. 2001). The colony encompasses

1. a clone of asexually derived feeding polyps (gastrozooids), with mouth and long tentacles;
2. two types of colony-protecting polyps (dactylozooids and tentaculozooids) with knoblike tentacles richly supplied with thread capsules but which cannot feed, and
3. reproductive polyps (gonozooids), with knoblike tentacles, unable to feed but which carry the reduced medusa buds representing the gonads and produce gametes.

In highly differentiated colonies, such as *Hydractinia* and to the same degree in siphonophores (e.g., *Physalia*—“Portuguese man-of-war”), a zooid is favored through kin selection,³⁰ the fitness of the clone as a whole being enhanced by the nepotic behavior³¹ of these non-reproductive components.

The same rules of kin selection hold true for the physiological interactions of germ-line stem cells and somatic cells conglomerated in germ-line stem cell-niches (Decotto and Spradling 2005; Kai and Spradling 2003; Ohlstein et al. 2004; Xie and Spradling 2000). It further helps to put the debate of whether PGCs are arising through epigenesis or preformation into a more balanced perspective (see above). Kin selection is actually the most plausible of three hypotheses that have been constructed to explain the origin and evolution of eusociality: In *parental manipulation* the parents’ personal fitness is raised even though that of some of the offspring is lowered, whereby *social mutualism* will evolve if a group of individuals can reproduce better than a single individual under the same circumstances. Both hypotheses do not appear adequate to account for the origin of colonial existence, whereas kin selection can (Hölldobler and Wilson 1990, p 180).

3.2

Hamilton’s Rule and the Evolutionary Criterion of Altruistic Behavior

Charles Darwin recognized levels of selection on a transspecific (= macro-evolutionary) scale by comparing the number of species within the same

³⁰ **Kin selection:** term coined by John Maynard Smith. A phenomenon of inclusive fitness, used to explain altruistic behavior between related individuals. Kin selection refers to changes in gene frequency across generations that are driven at least in part by interactions between related individuals.

³¹ **Nepotism:** favoring relatives because of their relationship rather than because of their abilities.

genus (Darwin 1975, p 239): “*This genus may have one, two or even more varying species. Any of the species may vary; but it will generally be those species which are most numerous in individuals & most diffused; & this shows that such species have already some advantages over the other inhabitants of the country. From our principle of divergence, the extreme varieties of any of the species, & more especially of those species which are now extreme in some characters, will have the best chance after a vast laps of time, of surviving; for they will tend to occupy new places in the economy of our imaginary country.*” This made Darwin the founder of ecology as well as evolutionary biology. On the other hand, survival of the fittest at the level of germ-line cells versus somatic cells in multicellular organisms (a contemporary domain of studying apoptosis) led Weismann to grasp the meaning of levels of selection. In 1964, William Donald Hamilton realized that genetic identity between individuals of non-clonal organisms is relatively rare compared to lesser degrees of genetic relationship. He proposed that the general criterion is that the product of the benefit of altruism (B) and the degree of genetic relatedness (R) be greater than the cost of altruism (C)³².

$$R \times B - C > 0 \quad (1)$$

When this criterion is met, then it is possible for selection to favor altruism. Most critical is the measure of genetic relatedness. In the diploid metazoans, such as humans, for identical twins the genetic relatedness is $R = 1$. For parents and offspring, the degree of relatedness is on average $R = 0.5$, as each parent contributes half of the genes to the offspring. For full siblings it follows $R = 0.5$ as well. For half-siblings, with only one parent in common it is $R = 0.25$. For first cousins it is $R = 0.125$ etc. These values probably account for J.B.S. Haldane’s anecdotic reply to the question of whether or not he would save a drowning man, if it meant losing his own life. Haldane replied: “*I’d lay down my life for two brothers, four half-brothers, or eight cousins.*” Thus, kin selection requires some degree of genetic relatedness.

As mentioned above many cnidarian colonies such as siphonophores, *Hydractinia* or clonal coral polyps are thought to be shaped by kin selection. One might not always be aware that coral reefs dominating tropical coast lines and deep sea corals inhabiting the colder deep waters of continental shelves and offshore canyons are made up of polyp colonies whose phylogenetic descent may have fallen under the rules of kin selection. Most biologists and even most entomologists are not aware of the fact that the recent insect fauna on earth has become predominantly social by means of kin selection. About one-third of the entire animal biomass of the Amazonian rain forest is composed of ants and termites. Along with bees and wasps they make up 75%

³² R = the genetical relatedness of the recipient to the actor, usually defined as the probability that a gene picked randomly from each at the same locus is identical by descent.

B = the additional reproductive benefit gained by the recipient of the altruistic act.

C = the reproductive cost to the individual of performing the act.

of the total insect biomass (Hölldobler and Wilson 1990). Especially the hymenopteran insects developed social systems. They encompass bees, wasps and ants and represent the largest order of insects with at least 125 000 species (for comparison, plants consist of only 290 000 species worldwide). Members of this insect order have an unusual system of sex determination. Fertilized eggs become females, and are diploid. Unfertilized eggs become males, and are haploid. *Hymenopterans* are therefore haplo-diploid. Haplo-diploidy, such as in the honey bee *Apis mellifera*, is important for the evolution of altruism and insect societies because it changes the landscape of genetic relationships within a species. Haplo-diploid full sisters (worker bees), who share both a mother (diploid queen) and a father (haploid drone), will on average have a coefficient of genetic relationship equal to 0.75. This is greater than the genetic relatedness of parent to offspring worker bees which remains 0.5. In simple words this means that a worker bee gets a greater benefit from facilitating the production of sisters rather than daughters. Nature has done so by evolving female worker bees into helpers of the queen in maintaining the nest rather than reproducing themselves. Therefore, a prediction that we can make about haplo-diploid societies, when females are diploid, is that they should frequently be organized into groups in which daughters help their mothers rear more daughters. Male bees (drones), on the other hand, are produced from unfertilized eggs. Therefore, there is no genetic relatedness between fathers and sons, and brothers and sisters are related with only $R = 0.25$. Therefore, drones are less related to other members of the family group in haplo-diploid superorganisms compared to conventional diploid systems. The beekeeper knows that drones exhibit little tendency to altruistic behavior, striving only to mate, never assisting their sisters nor brothers, and for that, they are quickly culled by their sisters after the mating season is over or by the beekeeper who removes the larger sized drone combs selectively. The link to Weismann's cognition of the hierarchy of selection could not be better formulated as by John Beard, 1904: "*In Nature, then, the multicellular individuals or Metazoa are the drones of the hive—only retained until their purpose is carried out. Their task is to harbor and nourish the unicellular organisms known as germ-cells during a certain portion of the life-cycle. This accomplished, their part is played. Weismann writes, 'es besteht eine vollkommene Continuität des Lebens'—Nature demands a continuous and constant succession of life, and this she can only obtain by the aid of unicellular organisms. The Metazoan individuals are but her tools!*" (Beard 1904).

In summary, there is a striking correspondence between the most organized *Hymenopteran* societies and the predictions of Hamilton's kin-selection theory. The take-home message is that haplo-diploid populations will frequently be organized into collectives where the daughters help their mothers to raise more daughters. Interestingly, in analogy to those somatic cells of any metazoan individual which retain the capacity to dedifferentiate into germ

cells, sterile worker bees can be transformed into fertile queens just by manually transferring worker bee larvae less than three days old into empty queen honeycombs where they are fostered with royal jelly. In the same way, when a hive lost its queen by accident fertile queens will be re-raised. Adult worker bees just reshape worker bee-honeycombs and nurture early worker bee larvae with royal jelly “dedifferentiating” and transforming them into fertile queens. The most extreme way of redefining the normal germ-line pathway is when worker bees are kept without a queen. This artificially stimulates them to lay haploid eggs. From these, fertile drones can be raised which are able to feed back their genome into a new, experimental bee colony. Thus, there are dead-end streets for the germ line, which occasionally can be re-opened for transit traffic on the cellular level as well as on the level of whole organisms.

4

Maintaining Sex in Metazoans

Only geneticists can properly answer the question, ‘Is sex necessary?’ ... it should be recognized that the core of the idea ... was conjured up long ago by the genius of Weismann.

(Muller 1932)

4.1

Introduction

To my knowledge, Charles Darwin did not discuss the question as to *why* there is sexual reproduction. His major works between 1856 to 1859, i.e., “*The Origin of Species*” and its unpublished predecessor manuscript both focused on natural selection but not on sex (Darwin 1859, 1975). His second grand opus of 1871 then concentrated on sexual selection as a distinctive evolutionary force (Darwin 1871). Darwin here did not treat the question as to *why* there is sex, but he recognized *sexual selection* as a form of selection contributing to many biological phenomena which deserve separate treatment on their own. He was deeply concerned with themes like

- a) inheritance limited by sex,
- b) relative proportions of sexes (i.e., genders),
- c) secondary sexual characters,
- d) sexual and natural selection, contrasted,
- e) effects of the loss of sexual characters
- f) limitations of sexual characters,
- g) sexual differences in man,
- f) sexual similarity, and last but not least
- g) an explanation of sexual selection (Darwin 1871).

Thus, between Darwin's interests in selection in relation to sex and our present day understanding of sexual reproduction (i.e., involving haploid-diploid cycles, gamete dimorphisms, zygotes, germ cells, somatic cells and expressional dosage compensation of sex chromosomes) we can ask: "How did the question arise as to *what use does sex have and how it is maintained?*"—What are the answers that have been given? It is obvious that sex is widespread and dominates all multicellular life forms, and therefore asexual constellations or life histories incorporating parthenogenesis in nature appear to be rare, but nevertheless they do exist (I. Schön, D.K. Lamatsch and K. Martens, this BOOK). A decade after Darwin's work on sexual selection, August Weismann's early 1884 article was still fuzzy about the issue, but asexual, parthenogenetic reproduction, keeping an eye on honey bees, already played a relevant role: He first started his classic *continuity of the germ plasm* essay with the concept of the germ line where he recognized its potential immortality (Fig. 3B) (Weismann 1892; see also Weismann 1893³³). Then, he adjoined a section dealing with the meaning of the "*Richtungskörperchen*", i.e., the German word for the dwarfed meiotic products we call today *polar bodies*. In the third section Weismann used these to make a case (pp 83–84) as to whether the polar bodies were the *male part lost from an egg*. At the time it was believed by some authors that only if an egg would receive a new *male half* through fertilization development would continue. Balfour even noted that eggs acquired the function to form polar bodies *in order to prevent parthenogenesis*. Vice versa, an egg which would not eject its *male half* (the polar bodies) would be able to develop parthenogenetically without fertilization. Weismann rejected this conclusion that parthenogenesis is nothing else but keeping the *Richtungskörperchen* within the egg. He assumed, although he admitted simultaneously that this issue was not yet fully solved, that the potential for maturation of an egg is the same with or without polar bodies. And he used the production of honey bee drones as proof, where the same egg can either be fertilized producing a female worker bee or a queen or if unfertilized will develop parthenogenetically to produce a drone.

Then, 1889, Weismann used the argument that sex functions to provide variation for natural selection to act upon (Weismann 1889). According to Austin Burt and Stephen Gould, Weismann's explanation was widely accepted for much of the first half of the past century, but arguments of G. C. Williams raised doubts (Williams 1966). Williams rejected all claims for group selection, so long as Darwinian organismic selection (i.e., natural selection) can portray the same phenomenon in principle (Burt 2000; Gould 2002). This debate led Bell to enthusiastically call sex "The Masterpiece of Nature" and identifying its function was seen as "the queen of problems of evolutionary biology" (Bell 1982, p 19; I. Schön, D.K. Lamatsch and K. Martens, this BOOK).

³³ "Germ-plasm constituting the immortal reproductive substance"

4.2

Emergence of Diploidy

1893 Weismann presented details in part III of his book *“The Phenomena of Heredity Resulting from Sexual Reproduction”*. In a chapter on *“Modification of the Germplasm caused by Amphimixis³⁴”*, he drew attention to the necessity of a halving of the germ-plasm (Weismann 1893, pp 230–239). This brings us back to the biofilms speculated on above, and the origin of diploidy. While it may never be possible to reveal the real origin of diploidy, its advantages can be accounted for. James F. Crow and Motoo Kimura dealt with this question in their *Introduction to Population Genetics Theory* (Crow and Kimura 1970): *“The evolutionary advantages of recombination can be obtained in species that spend most of their lives as haploids as well as in those that are predominantly diploid. Yet there has often been evolution toward diploidy. What is the reason?”*

Crow and Kimura balanced three arguments in favor and disfavor of diploidy which I can do no better than quote unabridged:

1. *“At a first glance it would appear that there is an obvious advantage of diploidy in that dominant alleles from one haploid set can prevent the expression of deleterious alleles in the other. However, as soon as a new equilibrium is reached the mutation load will be the same; in fact, it will be twice as large unless the mutants are completely recessive. From this standpoint diploidy is a disadvantage, not an advantage. However, when the population that is previously haploid suddenly changes to diploid there is an immediate advantage. To be sure, when the equilibrium is reached the advantage is gone; but by this time the diploid condition may be established and there is no way of going back to haploidy without all the deleterious effects of exposing recessive genes. So it may be that diploidy is not conferring a lasting benefit, but is the result of a temporary advantage that cannot easily be gotten rid of.”*
2. *“(Another obvious advantage) is overdominance³⁵. If such loci are common, there is a genuine advantage to diploidy provided such effects are important enough to compensate for greater mutation load from partially dominant loci.”*
3. *“A second possibility is the protection it affords from the effects of somatic mutation. The zygote in a diploid species or the gametophyte in a haploid plant may have approximately the same equilibrium fitness, but the effects of somatic mutation would be quite different. Diploidy would protect against recessive, or partially recessive, somatic mutants. If the soma were large and complicated, as in higher plants and especially animals, a diploid*

³⁴ *Amphimixis* is the union of gametes in sexual reproduction

³⁵ **Overdominance**: alternative term for heterozygote advantage, a condition where the phenotype of the heterozygote is fitter than the phenotype of either homozygote. Also similar to heterosis effect.

soma may provide a significant protection against the effects of recessive mutants in critical cells."

(Crow and Kimura 1970)

Thus the transitory advantage of diploidy which permits dominant alleles to suppress deleterious effects of recessive mutations will become irreversible as the return to haploidy would involve fitness reduction. The change from haploidy to diploidy therefore is a one-way road without return—an example of irreversibility in evolution (Bull and Charnov 1985). The other advantage of diploidy is the protection of somatic cells from mutational stress during the lifetime of an individual. Diploidy, and even more polyploidy help to diminish the negative pleiotropic effects of somatic mutations. Further, diploidy and polyploidy guarantee efficient and error-free repair of double strand breaks³⁶ occurring frequently during the cell cycle in every genome (see, for example, Adams et al. 2003). This kind of advantage is to the individual and the action of selection is more direct than that favoring sexual reproduction (Crow 1994).

Once diploidy arose, genomes required to be halved to reestablish haploid gametes (Weismann 1889), which subsequently prepared the ground for the evolution of meiosis. As the advantages of diploidy, the intrinsically coordinated and obligatory halving of germ-line genomes (meiosis), the segregational assortment of chromosomes, and the use of multiple copies of DNA strands in mending mutational ravages had been established, the emerged complex became nothing else but what we call sexual reproduction. James F. Crow concluded, that the most significant value of sex lies in the ability to reassort existing genes as the environment changes and in the elimination of harmful mutations (Crow 1994; Crow and Kimura 1965a; Kondrashov and Crow 1991).

4.3

Recombination as a Means to Fix Beneficial Mutations

Mendelian inheritance combined with the meiotic reduction divisions today is omnipresent in plants and metazoans, attesting to their great evolutionary value. Once established in diploid populations, be it unicellular or multicellular, sexual reproduction assured the evolutionary advantage of recombination and segregational chromosome assortment as represented in two principal ideas:

1. recombination makes it possible for favorable mutants that arose in different individuals of the same population to get into a single individual (Fisher 1930; Muller 1932).

³⁶ For example, the soma of *Drosophila* larvae is mainly polyploid. Compared to largely diploid adults, these larvae encompass a high degree of resistance towards deleterious effects of P element-transposase triggered DSBs. The main mechanism for DSB repair here is SDNA.

2. recombination permits the species to respond more effectively to an ever-changing environment (Sturtevant and Mather 1938; Wright 1931).

Relevant for the first of the two principal ideas, Schön et al. review the lessons to be learned from ancient asexual populations (this BOOK). The idea of a model dealing with asexual populations had been first proposed by Muller (1932). Muller recognized that in an asexual species (such as non-ancient asexual stick insects or ancient bdelloid rotifers) mutations that arise in different individuals cannot be combined in one. This means that by integrating Weismann's germ-line idea, sexual populations are mixing their germ-line lineages throughout time by recombination and assortment while asexual populations evolve in separate parallel germ-line lineages. According to Muller, two beneficial mutants can be incorporated into an asexual population only if they occur within descendants of the same germ-line lineage in which the first mutation took place. Crow and Kimura note that the limiting factor is the time required for the descendants of the first mutant to increase to such numbers that the establishment of a second mutant becomes reasonably probable. In a sexual Mendelian population all the favorable mutants that occur during this interval can be incorporated. An asexual system will be as efficient as a sexual system only if the mutation rate is so low, the selective advantage of the mutant so great, or the population so small that the first mutant is established before another favorable mutant occurs (Crow and Kimura 1965a; Crow and Kimura 1965b; Crow and Kimura 1970). Figure 6 illustrates the concept as published in Muller's classic paper (Muller 1932). Its qualitative conclusion comes down to the following: The relative advantage of sexuality will be greatest when the population is large, when the mutation rate is high, and when the selective advantage of the mutant is small. Crow and Kimura conclude that the advantage of sexuality is greatest when the system is evolving by very minute steps in a large population.

The second of the two principal ideas, i.e., that recombination permits the species to respond more effectively to an ever-changing environment was put forward during the 1930s (Sturtevant and Mather 1938; Wright 1931). Copying the tradition of Crow and Kimura (Crow and Kimura 1970) I like to quote directly from Sturtevant and Mather's article: *"The simplest system that we have been able to devise, having the required property of favoring recombination, is as follows: Two gene pairs, A, a and B, b, exist in a population subjected frequently to three different sets of environmental conditions, D, E and F. Condition D favors A but acts unfavorably on B, whereas E favors B but lowers the frequency of A. These, if properly adjusted as to intensity of selection and number of generations over which they operate, will insure the perpetuation of both allelomorphs at each locus. Recombination will not, however, be of importance. For this it seems necessary to introduce the third condition, F, favoring the combination AB (with or without a similar effect on ab), but acting adversely on the single types A and B. Under such conditions it would appear that there*

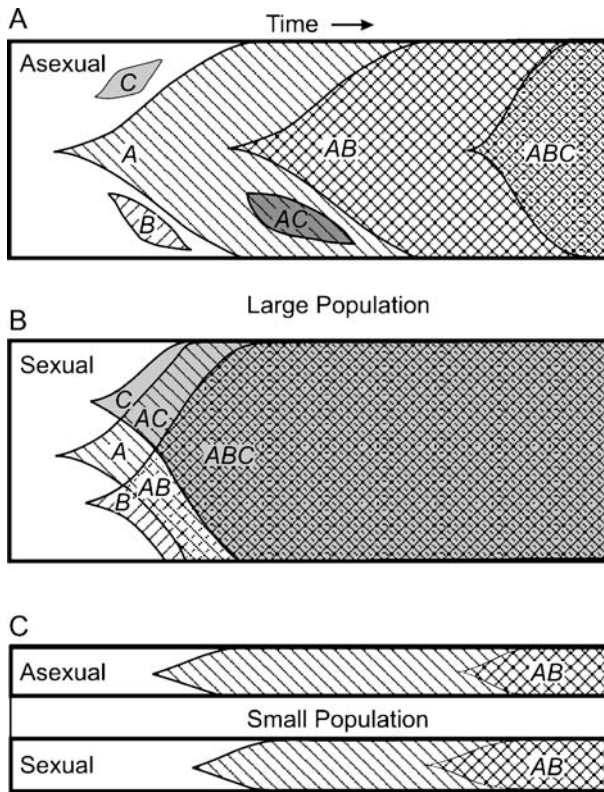


Fig. 6 Effects of recombination on evolution in sexual and asexual populations. Three mutations occur in three different germ-line lineages. The mutants A, B and C, are all advantages. **A** In a large asexual population when all three mutants arise at about the same time only one usually prevails. Here, A is more fit than B or C; A perhaps may also just have been luckier in happening to occur in an individual that was for other reasons unusually well adapted. B can be incorporated only when it occurs in an individual that already contains mutant A, and this will not happen on the average until the descendants of the original A mutant have grown to numbers roughly reciprocal of the mutation rate. C is finally incorporated as well, but only after AB individuals have reached significant numbers. Thus, in an asexual population, mutations are incorporated only in series of the germ-line lineage that survives because of unusual fitness or by chance. The spread of a mutation is accomplished by clonal distribution only. **B** The large sexual population incorporates mutants in separate germ-line lineages in parallel. Recombination and segregational assortment quickly lead to combination of favorable mutations within the same germ-line lineage and guarantee that they are readily spread in the population. **C** Small sexual as well as small asexual populations do not differ significantly in the time new mutants are spreading throughout the population. Thus, the relative advantage of sexuality will be greatest when the population is large, when the mutation rate is high, and when the selective advantage of the mutant is small—for each of these favors the occurrence of more mutants that can be incorporated in series (based on Muller (1932), adapted from Crow and Kimura (1970)). A more quantitative treatment was published by (Crow and Kimura 1965a, 1965b)

will be a selective action favoring recombination, of the order of magnitude of the selection of the AB type, as opposed to Ab or aB, under condition F. Other combinations of two or more loci may be exerting similar action in similar or dissimilar environmental conditions, and so the net effect will probably be to favor recombination for the majority of loci under the majority of condition changes.”

(Sturtevant and Mather 1938)

It remains undecided whether the Fisher–Muller concept or the Wright–Sturtevant idea is more important. Probably, both are valid and everything depends on limiting factors such as the changing environment or the chance occurrence of new mutants effecting the future of a tinkering evolution (Jacob 1983).

4.4

Recombination: Quantum Dimension Versus Ecological Dimension

Falaschi recently noted: “Until now, biologists have taken for granted that the classical approach is sufficient for their studies, and the macromolecules involved are treated as tiny, deterministic objects, with odd shapes and peculiar sticky properties on their surfaces. But is this really adequate? To be aware of the possible relevance of the quantum dimension in biology, just consider the resolution of a Holliday junction, the crossover-point between two homologous chromosomes during recombination: whether one particular DNA phosphodiester bond is broken in place of another one decides the outcome of a recombination event, that is, it may decide whether the organism issuing from the event is alive or dead; in other words, for a certain time, before the collapse of the wave packet³⁷, there is a superposition of an alive and dead organism.” (Falaschi 2007). For example, the addressed quantum level effects have been analyzed biophysically in the active catalytic center of myosin proteins with regard to the ATP hydrolysis mechanism. Here it was found by using quantum mechanical/molecular mechanical reaction path calculations that the specific reaction pathways can vary in the activation mechanism of the attacking water molecule and in the way the hydrogens are transferred between the heavy atoms (Schwarzl et al. 2006). Falaschi uses the resolution of a Holliday junction (HJ) with good reason. Depending on whether a detrimental point mutation is located left or right to a HJ, junction-migration by just a nucleotide up or down relative to the junction decides whether the mutation becomes manifested or not in one of the next generation’s genome copy. In the germ line it has both, the potential to turn a cell into becoming tumorous, and more relevant to affect an individual of the next generation; in the

³⁷ In physics, a **wave packet** is an envelope or packet containing an arbitrary number of wave forms. In quantum mechanics the wave packet is ascribed a special significance: it is interpreted to be a “probability wave” describing the probability that a particle or particles in a particular state will have a given position and momentum.

soma it may trigger cancer³⁸. In combination with Muller's model (Fig. 6) the specifics of a HJ-resolution pathway likely become transferred to yet higher levels of complexity as recombination can have an effect on the frequency distribution of genotypes within a population. By and large, recombination commits the advantage of reducing the frequency of non-optimal combinations of mutations and can potentially increase the frequency of superior combinations. The specific path taken is initiated at the quantum dimension but selection acts on all levels. In accordance with Stephen Gould's delineation of the theory of hierarchical levels of selection (see Sect. 2.1, above) Christof Biebricher once noted: "*Biologists still debate the identity of the target of selection: is it an ecosystem, a subpopulation, an individual, a gene or merely a 'replicator unit' (Dawkins 1982)? There is no ultimate answer to this question: selection takes place at all of these levels. Which of the selection level dominates depends on the environment.*"

(Biebricher 1999)

From this we can conclude that there may be an interaction between the quantum dimension of recombination up to the ecological level. Thus, recombination governed by hierarchical levels of selection (see Sect. 2.1, above) is prone to govern all hierarchical levels of life—genes in cells, cells in organisms, organisms in demes, demes in species, species in higher clades.

4.5

Recombination as a Means to Eliminate Detrimental Mutations

The further we distance our models of the evolution of genetic systems from the quantum level, the more complex and speculative they become. There are not only diploid or haploid organisms like us or *Bryophyta*, or haplo-diploid species like the social honey bees, species with mixed reproduction, either cyclic parthenogens, or organisms with both sexual and asexual lineages do occur as well. There are also hermaphrodites and a whole range of intermediate processes between obligate sex and apomixis. To account for all this would go beyond the scope of this chapter. Ancient-asexual species are treated in the contribution of I. Schön, D.K. Lamatsch and K. Martens (this BOOK).

However, recombination as a means to eliminate detrimental mutations requires a brief overview: Muller's model (Fig. 6) delineates that recombination may or may not speed up adaptive evolution depending on population size. His model pioneered the understanding of the long-term advantage of sex at the level of mendelian populations. However, under this model, the costs of meiosis may not have been enough accounted for as only long-term advantage but no short-term advantage seems to warrant the meiotic system. And yet, Darwinian evolution should explain the existence of meiosis by

³⁸ Primarily, a tumor will only harass the individual affected, although certain tumor cells can also become infectious, as documented for Tasmanian Devil's cancer (Pearse and Swift 2006).

cumulative selection, i.e., small, undirected variations that are channelled by selective pressures, resulting, after long periods of time, in all complex phenomena representing meiosis. Darwin notes: “*Let an architect be compelled to build an edifice with uncut stones, fallen from a precipice. The shape of each fragment may be called accidental; yet the shape of each has been determined by the force of gravity, the nature of the rock, and the slope of the precipice, events and circumstances, all of which depend on natural laws; but there is no relation between the laws and the purpose for which each fragment is used by the builder. In the same manner the variations of each creature are determined by fixed and immutable laws; but these bear no relation to the living structure which is slowly built up through the power of selection.*” (Darwin 1868, pp 248–249). Therefore, with regard to the evolution of meiosis, in addition to Muller’s model, other factors must be accounted for as well. In reality, deleterious mutations prevail, mobile populations migrate between habitats, environments vary temporarily and spatially, population size is large or small or fluctuates, and sexuality can alternate with asexuality within the same population of many species (e.g., aphids).

In environmental **deterministic models**, selection does not act on new mutations, but on existing genetic variation, by changing the distribution among all the possible combinations. Let us combine the classical Bergmann’s Rule³⁹ as a starting point (Rensch 1947) and a textbook example (Futuyma 1998; Maynard Smith 1980): we assume that the alleles A, B, C, D increase body size additively while alleles a, b, c, d decrease it. Stabilizing selection for intermediate size reduces the variance and creates negative linkage disequilibrium, so that combinations such as AbCd and aBcD are present in excess. If selection occurs in arctic regions so that larger size is favored (Bergmann’s Rule), combinations such as ABCD would not exist in a hypothetical asexual population, but they can arise rapidly in a sexual population. This provides another long-term advantage to sex as well as a short-term advantage as well, because sexual parents are likely to leave more surviving offspring than asexual parents. Forgotten, at least by this otherwise so distinguished textbook, the idea of this advantage of sex goes directly back to August Weismann as well. Austin Burt cites Weismann (Weismann 1904, p 223): “*The communication of fresh ids⁴⁰ to the germ plasm implies an augmentation of the variational tendencies, and thus an increase of the power of adaptation. Under certain circumstances this may be of direct advantage to the individual which results*

³⁹ **Bergmann’s Rule** is a principle that correlates environmental temperature with body mass in warm-blooded animals. It asserts that within a species, the body mass increases with latitude and colder climate. Among mammals and birds, individuals of a particular species in colder areas tend to have greater body mass than individuals in warmer areas. For example, the southern moose of Yellowstone park are both the smallest of their kind and the southern most ones. Their fellows living in the arctic in Denali Natl. Park encompass much more mass. The reason given by zoologists is the smaller relative surface of larger animals favorable in harsher temperature conditions compared to a relative large surface of smaller animals.

⁴⁰ genes

from the amphimixis, but in most cases the advantage will be only an indirect one, which may not necessarily be apparent in the lifetime of this one individual, but may become so in the course of generations and with the aid of selection. For amphimixis must bring together favorable as well as unfavorable variations, and the advantage it has for the species lies simply in the fact that the latter are weeded out in the struggle for existence, and that by repetition of the process the unfavorable variational tendencies are gradually eliminated more and more completely from the germ-plasm of the species" (Weismann 1904, p 223). Austin Burt interprets Weismann's almost prophetic insight to mean that sex (and by extension, (meiotic) recombination) does not function to increase mean fitness directly. Rather, Weismann assumed that sex increases the variance of fitness, and thus the response to selection, and mean fitness after selection (Burt 2000). Weismann's mechanism appears to underlie most of the formal population genetic models aforementioned for the evolution of sex and recombination. Thus, under this premise, sexuality and asexuality are traditionally thought to be differently effective in ridding populations of disadvantageous mutations (but see also Omilian et al. (2006); or I. Schön, D.K. Lamatsch and K. Martens, this BOOK). Two important models illuminate how detrimental mutations are eradicated: Muller's ratchet and Kondrashov's hatchet hypotheses.

4.5.1

Muller's Ratchet

Muller's ratchet hypothesis starts with an asexual population where none of the individuals contains any detrimental mutation in its sequence, and where all individuals are of superior fitness. After some time deleterious mutations at multiple loci arise and create a mutant spectrum of germ-line genotypes carrying 0, 1, 2, ..., m mutations. Over time, individuals with zero mutations become rare and disappear from the asexual population entirely as its members continually experience new mutations. The zero class may also get lost just by chance, especially from small populations. The remaining germ-line genotypes all encompass at least one harmful mutation. In the long run, the one-mutation class is lost, and all remaining individuals carry at least two mutations. Individuals of the zero- and one- mutation class cannot be regenerated in an asexual population. Only the ancient replicative DNA-repair and gene conversion mechanism termed synthesis-dependent strand annealing (SDSA) could, in a diploid genome, restore the favorable back mutation (J. Haber, this BOOK). However, whether SDSA plays a central role to restore lost genomic information in ancient asexual populations needs to be experimentally specified in the future. So far, Muller's ratchet hypothesis assumes that the loss of superior genotypes is an irreversible process—a ratchet. Asexual populations accumulate inferior genotypes over time, lowering population size, which in turn facilitates to increase the rate

of the least mutation-laden genotypes to get lost by genetic drift⁴¹. The accelerated decline in fitness is called “mutational meltdown” (Gabriel et al. 1993; Lynch et al. 1993, 1995; Lynch and Gabriel 1990).

On the other hand, recombination and segregational chromosome assortment in sexual populations followed by selection of the best adapted genotype, reconstitute the least mutation-laden genotypes. In sexually reproducing organisms non-recombining chromosomes or chromosomal regions like, e.g., the mammalian or *Drosophila* Y chromosome, might also undergo Muller’s ratchet. The Y chromosomes appear to repair double-strand breaks by means of template-assisted recombinational repair at its complementary sister-chromatid sequences but this “self-recombination” does not neutralize this chromosome’s tendency to undergo Muller’s ratchet. In *Drosophila*, males live on perfectly healthy without their Y chromosome. However, some essential germ-line-specific components necessary for spermatogenesis appear to remain associated with this chromosome such that XO males are infertile (Hennig 1967, 1985; Hennig et al. 1989).

4.5.2

Kondrashov’s Hatchet

Kondrashov’s hatchet is sometimes called the deterministic mutation hypothesis. It is a model, in which deleterious mutations are assumed to take on strong synergistic effects (Kondrashov 1988, 1993; Kondrashov and Houle 1994; Kondrashov and Kondrashov 1999) (Fig. 7). The model applies to large populations, no genetic drift⁴¹ is required. The majority of harmful mutations are only slightly deleterious. The hypothesis assumes that harmful mutations interact, such that each loss of a genotype rids the population of more than one deleterious mutation, and fewer genetic deaths are required to improve the population’s mean fitness than would be required if the mutations were eliminated one by one. The model assumes a threshold number of mutations (T) behind which the fitness of the respective asexual individual is greatly lowered (Fig. 7). Most importantly, a model like this needs to be tested in a suitable experimental system. Ancient or recent asexual species could provide the experimental material.

5

Finale

Richard Owen (1849)—now best known for purportedly having coached Archbishop Wilberforce prior to his notorious attack against Charles Dar-

⁴¹ random changes in gene frequency especially in small populations when leading to preservation or extinction of particular genes

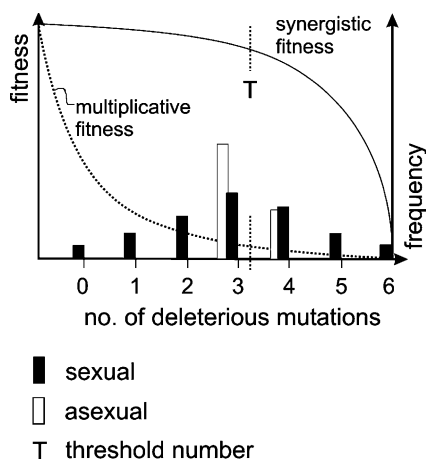


Fig. 7 Kondrashov's hatchet hypothesis. Deleterious mutations are eliminated more rapidly from sexual than from asexual populations. *Upper curve* represents the synergistic epistasis in Kondrashov's model. *T* delineates the threshold where the fitness in asexual populations breaks down greatly. From here on, each mutation has a disproportionately large effect on the organism's fitness. *Lower dotted curve* shows how, in theory, fitness would decrease if growing numbers of mutations would multiplicatively diminish fitness. *Bars* represent the portion of individuals representing a particular genotype with *n* mutations. *White bars* represent proportions in an asexual population. *Black bars* represent occurrence numbers in a sexual population. Before selection in any given population, the frequency distribution of mutations per individual is broader in a sexual population, due to recombination, than in the asexual one. Since the greater number of mutations are carried by individuals with more than *T* mutations in the sexual than in the asexual population, the frequency of each detrimental mutation is reduced faster in the sexual population

win but defeated by Thomas Huxley at the British Association meeting of 1860—was the one to whom priority might be given for suggesting that the germ-cell line is continuous from one generation to the next (Tobler 1986). August Weismann, however went much further. He recognized not only the deep conceptual significance of the germ-line idea but that another kind of selection, different from Darwin's natural selection, made an impact on the level of cells. Weismann called it "*germinal selection*"—that is the segregational differentiation between somatic and germ cells. In our time, research dealing with this issue falls into the realms of developmental biology. By understanding the influence of germinal selection Weismann recognized that if selection existed below the organismal level, then the same logic implies the existence and potency of supraorganismic levels of selection as well (Gould 2002).

In the light of these insights, together with our present drive to comprehend the origin and evolution of meiosis, we may be able to classify the following controversial debate. Austin Burt discounted Bernstein et al.'s sug-

gestion that “*meiotic crossing-over is just an incidental by-product of DNA repair processes*” (Burt 2000, p 347). I suspect that here, a misunderstanding may be on hand. Stephen Gould noted, the hierarchical theory of selection recognizes many kinds of evolutionary individuals (Gould 2002, p 674). Carol and Harris Bernstein seemed to adhere the origin of sex and meiosis to a very basic evolutionary individual, i.e., DNA repair. In their book on Aging, Sex, and DNA Repair (Bernstein and Bernstein 1991, p 292) they argue: “*Our view is similar to Dougherty’s (Dougherty 1955) in assuming that sex arose early in evolution as a recombinational repair process and that it has had a continuous evolutionary history (Bernstein et al. 1981, 1984, 1985a, 1985b, 1987, 1989). We differ somewhat with Dougherty in assuming that RNA, rather than DNA, is the primitive genetic material and, more strongly, in considering the acceleration of evolutionary improvement to be a by-product rather than a selective advantage of sex.*” Burt continues his criticism on Bernstein’s statement: “*The most pressing problem with the idea is that (meiotic) crossing-over involves only one of the two sister chromatids of each chromosome, and so under the hypothesis only one of them should be damaged. However, if this is the case, why not just repair it with the sister chromatid? This is what usually happens in mitotic cells if damage occurs between DNA replication and cell division (Kadyk and Hartwell 1992). However, in meiotic cells, the preferred template for “repair” is the homolog, not the sister chromatid (Schwacha and Kleckner 1994, 1997). This is as expected if the function of meiotic crossing-over is to produce recombinant chromosomes, but not if it is simply to repair broken chromosomes.*”

To me the debate appears a little like shadow boxing. The Bernsteins, perfectly legitimately, address the likelihood of the origin of meiosis from ancient replicative DNA repair systems (D. Penny and R. Egel, this BOOK). They do not specify as to how new mechanisms arose and contributed to meiosis in a phylogenetic setting. A fairer discussion on this issue was presented in the fine textbook of Futuyma (1998, p 608). “*Kondrashov (1993) distinguished hypotheses that propose some immediate benefit to the individual organism’s progeny from hypotheses that invoke variation and selection.—The leading hypothesis for an immediate benefit of recombination is that molecular recombination facilitates repair of damaged DNA (Bernstein and Bernstein 1991). According to this model, breaks and other lesions in a DNA molecule can be repaired by copying from an intact sequence on a homologous chromosome. According to this hypothesis, the formation of new gene combinations is a by-product of the molecular mechanism of DNA repair, not the raison d’être⁴² of recombination or sex. Critics of this hypothesis (Kondrashov 1993; Maynard Smith 1988) argue that it fails to explain the elaborate mechanism of meiosis and syngamy, and that DNA repair does not require these mechanisms; permanently diploid or polyploid apomicts, of which there are many, could also repair*

⁴² right to exist

damage this way. Most evolutionary biologists would grant that the origin of recombination may have been due to its role in DNA repair, but believe that the evolution of meiosis and distinct mating types or sexes, and the maintenance of sex in most species, must be attributed to other causes involving variation and selection.”

The latter sentence seems best to represent Weismann’s early recognition of levels of selection, and thus, to grasp the origin of meiosis as a multilevel evolutionary phenomenon. Only in this way we should be able to comprehend the various peculiarities of so different meiotic systems like *Drosophila* spermatocytes that undergo meiotic divisions without crossing over, *Saccharomyces cerevisiae* reduction divisions determined by two HJs and *Schizosaccharomyces pombe* encompassing only a single HJ (Cromie et al. 2006; G. Cromie and G. R. Smith, this BOOK). The most basic level addressing perhaps the most ancient and fundamental level of selection and replicative recombination is that of the smallest replicators, i.e., viruses and transposable elements. This is the realm of Schuster’s and Eigen’s quasispecies theory explaining quantitatively the boundary, where evolution works best (Eigen 1988, 1992). Table 1 summarizes the multiple hierarchies of selection with

Table 1 Hierarchy of levels of selection

	Level of selection	Founder	Name of theory	Biological entity
∞	general all levels	Charles Darwin	natural selection	molecules to ecosystems
1	non-cellular	Manfred Eigen & Peter Schuster	quasispecies	viral replicators or transposable elements
2	cellular	August Weismann	germinal selection	germ line/soma
3	organismic	Charles Darwin	natural selection sensu stricto	organisms within populations
	organismic	Charles Darwin	sexual selection	male, female individuals
4	supraorganismal, colonial superorganisms	Donald Hamilton	kin-selection	e.g., insect societies, cnidarian colonies, eusocial sponge-dwelling shrimp, mole rat societies
5	population	Fisher–Muller Wright–Sturtevant–Mather	advantage of sex & recombination	populations within species
		Muller Kondrashov	Muller’s ratchet Kondrashov’s hatchet	asexual populations and eroding sex chromosomes

Darwin's natural selection overseeing all of them. The series begins on a level of non-cellular viral replicators accounted for in Schuster's and Eigen's quasispecies model (Eigen 1988, 1992), continues over August Weismann's germinal selection hypothesis, extends over Darwin's strategic theory addressing selection mainly at the level of organisms within populations (Darwin 1859), but later addressing sexual selection where males competed versus males and females versus females for reproductive success within populations (Darwin 1871). Next, the series steps up to colonial superorganisms such as insect societies (Hölldobler and Wilson 1990), cnidarian colonies (Frank et al. 2001) or eusocial sponge-dwelling shrimp (Duffy 2003), which Hamilton explained by kin-selection (Hamilton 1964). The grand population theories included the attempt to answer *why* sex is maintained in plants and metazoans. It was addressed in mathematical population theory by several of the neodarwinist giants such as Sewall Wright (1931) and Fisher's genetic theory of natural selection (Fisher 1930), Muller's ratchet or Kondrashov's hatchet being only small but important elements of the grand issue surrounding the theory of the germ line and maintaining sex in metazoans. Keeping the quantum dimension in mind, future research will have to tackle the broadly relevant issues as to what relative degree in a quantitative sense the various key mechanisms of recombination, DNA repair, transposon activity, epigenetic transmission and meiotic segregation contribute to the evolution of organisms. Only systems biology, independent of first and foremost phenomenologically dominated concepts, will be able to succeed in this direction in a balanced way. The guiding insights – we should always be aware of—are indeed deep-rooted in the ingenious cognition processes of Charles Darwin and August Weismann who laid the foundation for the theory of hierarchical selection.

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Lessons to Learn from Ancient Asexuals

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Abstract This chapter reviews current hypotheses on the prevalence of sex in the eukaryotic world and presents four examples of putative ancient asexuals. We evaluate theoretical and practical concepts on how to demonstrate long-term asexuality. These are either derived from classical biological research (e.g. absence of males in fossil or recent populations) or from molecular biology (e.g. the Meselson effect, presence of functional transposable elements). Testing for the presence of conserved meiotic core proteins is a novel and especially promising way to verify ancient asexuality. We further re-evaluate statistical methods that utilize existing DNA sequence information on how to test for meiotic recombination. Molecular mechanisms counteracting the accumulation of deleterious mutations might be the most important avenue for ancient asexuals to persist in long, evolutionary time frames. Such mechanisms are reassessed and linked to molecular data from putative ancient asexuals. In particular, the purging of deleterious mutations by ameiotic recombination appears to be more effective than previously anticipated.

Abbreviations

BAC	bacterial artificial chromosome
DSB	double strand break (in DNA)
EF	elongation factor
FISH	fluorescent in situ hybridization
GPG	general purpose genotype
<i>hsp</i>	heat shock protein
LTR	long terminal repeat
myr	million years
PCR	polymerase chain reaction
TE	transposable elements
UTR	transcribed untranslated leader region

1

The Paradox of Sex

For the vast majority (95%) of eukaryotes, sex¹ is an essential part of their life cycle. Sexual reproduction (see Fig. 1) alternates between meiosis and fertilization or syngamy. Its most important consequence (see below) is producing

¹ Synonymous term preferred by Kondrashov is amphimixis (Kondrashov 1993).

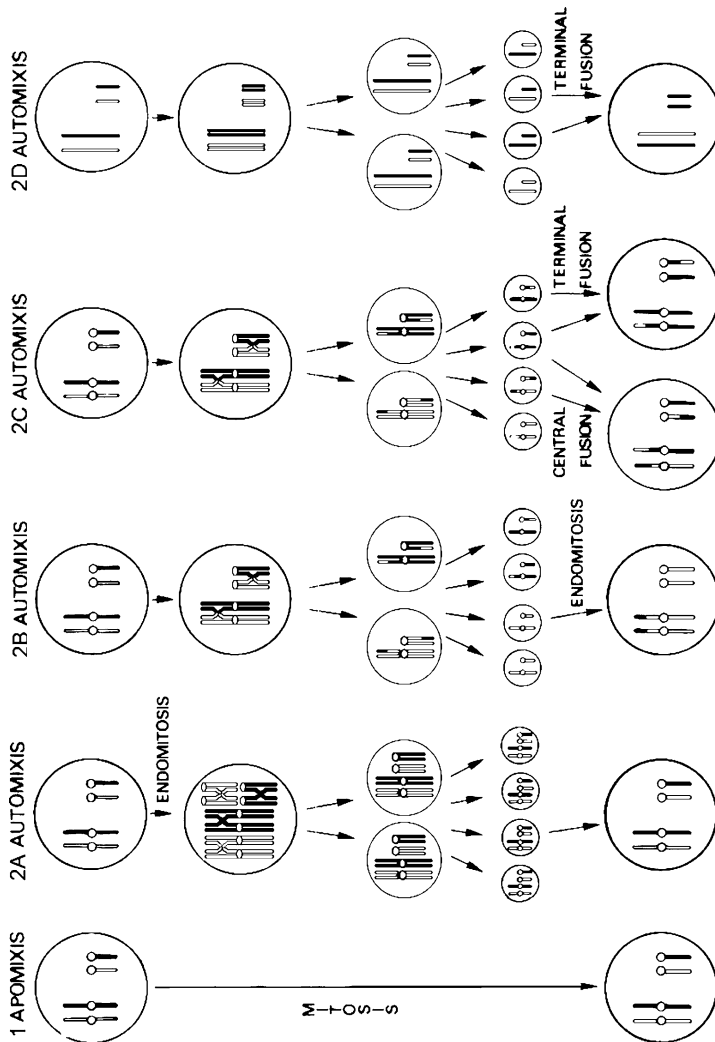


Fig. 1 Major modes of reproduction according to Butlin et al. (1998) with modifications. *1 Apomixis:* Meiosis is replaced by mitosis. Offspring are genetically identical to their mother. *2 Automixis:* *2A* Meiosis is preceded by endomitosis and involves pairing of sister chromatids. Offspring are genetically identical to their mother. *2B* Meiosis is followed by endomitosis. The result is complete homozygosity but offspring are different from their mother. *2C* Meiosis is followed by fusion of the products to restore the diploid chromosome number. *Terminal fusion:* the egg pronucleus fuses with the second polar nucleus, which would equal an anaphase II segregation failure. Increased homozygosity except for loci between centromere and beyond cross-over. *Central fusion:* the egg pronucleus fuses with a second division product of the first polar nucleus. Increased homozygosity except for loci between centromere and cross-over. *2D* Inverted meiosis with holokinetic chromosomes. Homozygosity increases if combined with terminal fusion; offspring are different from their mother

offspring that are genetically different from each other and from their parents. Sex and meiosis can be replaced by apo- and automictic asexual reproduction (see Fig. 1) or parthenogenesis². Parthenogenesis is widely distributed throughout the animal and plant kingdoms suggesting that asexual reproduction has evolved repeatedly. Because most parthenogens still engage in sexual reproduction at some point during their life-cycle, one can also speak about a continuum from fully sexual (amphimixis) to fully asexual (apomictic thelytokous) reproduction depending on the frequency of both reproductive modes within and/or between generations.

Despite the ubiquity of sex, sexual reproduction is very costly in an evolutionary sense (Maynard Smith 1978). This gave reason to entitle this paradox “the queen of evolutionary problems” (Bell 1982). The so-called twofold cost (Maynard Smith 1998a) of sex can best be translated into “*males are an evolutionary luxury*”. Indeed, males do not contribute directly to the number of offspring in the next generation and all-female populations have twice the number of offspring daughters, even with identical fecundities in both reproductive modes. Also, beneficial gene combinations risk being broken up through meiotic recombination as fast as or even faster (Hurst and Peck 1996) than they can be formed.

However, it seems that this evolutionary luxury is at the same time an evolutionary necessity because more than 95% of all eukaryotic species undergo sexual reproduction, at least occasionally. The “paradox of sex” has kept the brightest minds of evolutionary biology busy and more than 25 hypotheses have been put forward (for an overview see, for example, Butlin et al. 1998; Butlin 2000; Kondrashov 1993; Van Doninck et al. 2004a). Amongst the *genetic* hypotheses³, the best known are the mutation accumulation theory by Kondrashov (1988, 1993) and Muller’s ratchet (Muller 1964)⁴. Both theories rely on the fact that long-term asexuals lose the ability to purge their genomes from deleterious mutations.

Whereas Kondrashov’s theories mainly depend on the overall deleterious mutation rate and genome size, Muller’s hypothesis is more oriented towards population genetics and describes the fate of mutation-free genotypes (and at later stages genotypes with few mutations) in a finite population. Because ancient asexuals inevitably lose the ability to restore genotypes with no or few mutations, Muller has compared this process to a ratchet that clicks every time that one of these genotypes with low mutational load is randomly lost from the population by genetic drift. This theory thus in-

² Parthenogenesis: thelytoky – progeny are all females; arrhenotoky – males arise from unfertilized eggs.

³ Genetic hypotheses are the focus of this chapter; readers being interested in other theories such as sib-competition, the tangled back theory or clade selection should refer to, e.g., Butlin et al. (1998) or Butlin (2000).

⁴ The idea of the ratchet was originally described by Muller (1964) whereas its quantitative aspects were investigated later by others (e.g. Felsenstein 1974; Haigh 1978; Kondrashov 1994; Gordo et al. 2002).

volves mainly stochastic processes. Eventually, all genotypes will be so heavily loaded with deleterious mutations that the population will become extinct. Small population sizes will speed up this process. They can be further diminished by the so-called mutational meltdown when high rates of deleterious mutations increase mortality (Gabriel et al. 1993). Kondrashov's theory predicts the same fate but invokes a different mechanism: in sexual populations, variation in the number of mutations is restored every generation enabling selection to remove individuals with several disadvantageous mutations faster and more efficiently than in asexual populations. This process is independent of population size and thus deterministic. At least in theory, negative epistasis⁵ could further increase the genomic mutation rate although evidence for this theory is still lacking (Barton and Charlesworth 1998; Peters and Keightley 2000).

It is still doubtful, however, whether ancient asexuals have sufficiently high net genomic rates of deleterious mutations to make these theoretical predictions work (see the review by Butlin 2002). As a result of exceptionally high population densities of most ancient asexuals (see below), Muller's ratchet is probably slowed down considerably. Only few papers so far found evidence for Muller's ratchet acting on intracellular symbionts (Moran 1996) and theoretical predictions reveal that our species should have died out 20 myr ago through mutations accumulating in our mitochondria (Loewe 2006)! The reproductive mode might even affect the mutational rate in mitochondria, where Paland and Lynch (2006) estimated deleterious amino acid substitutions to be four times higher in asexual than in sexual lineages of *Daphnia pulex*. Unfortunately, no clear data on the age of the asexual clones are provided in this study, which makes it doubtful whether similar patterns should be expected from true ancient asexuals.

Other hypotheses on the prevalence of sex originate from *ecological* fields and focus on the generation of new gene combinations that are subsequently exposed to changing environments. The Red Queen hypothesis was coined by Van Valen (1973) to explain background extinction in the fossil record. Hamilton et al. (1990) and Hamilton (1980) view these interspecific arms races (e.g. parasites and hosts, predator-prey or common interspecific competition) from a biological point of view and use it to explain the prevalence of sex: sexual hosts would be better equipped to deal with fast-evolving parasites. A recent paper (Otto and Nuismer 2004) has shown, however, that the Red Queen is only applicable to selected cases of asexuality and therefore cannot provide a universal explanation for the prevalence of sex. Another hypothesis, fluctuating selection, focuses on abiotic changes of the environment (e.g. climatic fluctuations) (Maynard Smith 1978; Roughgarden 1991). However, similarly to the Red Queen, this hypothesis requires particular patterns

⁵ Negative epistasis is the significantly larger reduction of fitness in individuals carrying disadvantageous mutations at several loci as compared to the fitness if these mutations had independent disadvantageous effects.

of changing selection pressure making it very unlikely that it could provide a general explanation for the prevalence of sex.

More recently, several studies have reassessed the ecological cost of sex. Doncaster et al. (2000) and Pound et al. (2002) modelled competitive abilities of sexuals and asexuals at various carrying capacities. Depending on the chosen parameters, sexuals can drive out asexuals or coexist with them. Likewise, Scheu and Drossel (2007) could show that sexual reproduction prevailed when structured resources became scarce. These models seem to mirror the living world quite well but empirical studies are needed to verify their importance. The latter applies also to a recent publication by Otto and Gerstein (2006). They suggest that genetic drift is a powerful force in both small and large populations and could be the most straightforward explanation for the ubiquity of sex because it works with the required broad range of fitness curves and both negative and positive selection.

In conclusion, none of the 25 or more published hypotheses has so far been able to solve the paradox of sex unequivocally. The jury is still out to judge whether pluralistic models such as combining the effects of the Red Queen with the mutational load hypothesis (West et al. 1999) or spatial processes with mutation accumulation (Salathé et al. 2006) could do so.

To make matters even more complicated, the evolutionary consequences of asexuality will change dramatically with the kind of asexual reproduction (see above). If a certain species only occasionally reproduces parthenogenetically but experiences regular periods of sexual reproduction, then this mixed mode of reproduction will have genetic and genomic consequences very different from those of obligate asexuality. If the species in question have regular transitions between sex and asex and vice versa, the entire apparatus required for meiosis, spermatogenesis, mating etc. must remain functional. In ancient asexuals, all of these are, at least in theory, free to deteriorate or acquire new functions.

2

What is an Ancient Asexual?

The plausibility of long-term existence without sex (including the absence of meiosis) is still not widely accepted and this is reflected by the term “ancient asexual scandals” (Judson and Normark 1996). The mere existence of ancient asexuals defies the conventional wisdom on the advantages of sex. Some papers dismiss ancient asexual status with off-hand statements such as “all ancient asexuals are probably automictic” (Gorelick 2003) or “males will inevitably be found with more extensive screening” (Little and Hebert 1996). We will show that none of these statements holds true when closely inspected. To date, ancient asexuality is supported for three animal groups (see below why): bdelloid rotifers, darwinulid ostracods and certain groups

of oribatid mites⁶. The case of arbuscular mycorrhizal fungi is still debated (Pawlowska 2005) and will be more extensively discussed below. No higher plants are amongst the putative ancient asexuals; this might be a sampling effect or be owing to the Weismann's doctrine⁷. The separation between soma and germ line as found in animals might be an important prerequisite for ancient asexuality, and seems to be absent in plants. Passing somatic mutations on to the next generations will increase the effects of the mutational load and of Muller's ratchet. The phylum *Rotifera* holds worldwide about 1850 species (Segers 2002), mainly in aquatic and moist terrestrial habitats. It consists of three classes: the Seisonidea, *Monogononta* and *Bdelloida*. Whereas *Seisonidea* reproduce fully sexually, monogononts are cyclical parthenogens and bdelloids are ancient asexuals. Bdelloid rotifers comprise a group with more than 300 species for which males have never been reported. They reproduce through apomixis (Hsu 1956a, b). Bdelloid rotifers can undergo reversible desiccation during dry periods when the whole animal dries out. It seems that species from aquatic habitats recover less well than those living in mosses (Ricci 1998) and that age might affect survival and fecundity after desiccation (Ricci et al. 1987). The biology and ecology of bdelloids have been investigated from natural populations (e.g. Ricci 1983, 2001; Ricci et al. 1989) whereas the majority of molecular investigations has focused on selected species such as *Philodena roseola*, *Adineta vaga*, *Habotrocha constricta* and *Macrotrachela quadricornifera* (Arkhipova and Meselson 2000; Mark Welch and Meselson 1998, 2000; D. Mark Welch et al. 2004, J. Mark Welch et al. 2004), which are routinely cultured in the laboratory. Also, phylogenetic reconstructions have been conducted (Mark Welch 2001, 2005) to place bdelloid rotifers in a wider evolutionary context and compare patterns of molecular evolution between the ancient asexuals and sexual relatives (Mark Welch and Meselson 2001). Recently, the first extensive sequence analyses from the mitochondrial COI gene have started to define clusters of natural bdelloid populations, which might be equated to species levels (Birky et al. 2005).

The *Oribatida* are a suborder of acariform mites with 10 000 described species (Schatz 2002), which nearly invariably occur in high densities of up to 425 000 individuals per square metre (Anderson 1978). The parthenogenetic species *Oppiella nova* might very well be the most common arthropod on earth (Norton and Palmer 1991). Oribatids are mostly found in forest soil, although they also inhabit other habitats such as trees (André 1984), lichens and mosses on rocks (Travé 1963), caves (Hippa et al. 1988; Bruckner 1995) and even arid deserts (Wallwork 1972; Wallwork et al. 1986). They mainly feed on leaf litter and fungi (Travé et al. 1996). About 10% of all oribatid mites

⁶ What is sufficient and ultimate proof for ancient asexuality? We consider the likelihood as very high that the three taxa mentioned above are indeed ancient asexuals. Others might still regard them as putative cases.

⁷ See the chapter by Lankenau in this BOOK.

are parthenogenetic (Norton and Palmer 1991; Norton et al. 1993). The *Oribatida* are divided into six groups, of which the *Desmonomata* have several families with approximately 400 parthenogenetic species and two families from the *Enarthronota* with another 330 parthenogenetic species (Subias 2004). They probably reproduce with automictic parthenogenesis (Taberly 1987a,b), which might involve a process of inverted meiosis (Wrench et al. 1994; Heethoff et al. 2006) although this remains to be confirmed. Research in the past has mainly focused on cytogenetics, life-history, morphology and allozyme screening (e.g. Norton 1994, 1998; Norton et al. 1997; Palmer and Norton 1992) but recently, also DNA-based (Heethoff et al. 2007; Maraun et al. 2003, 2004; Schäfer et al. 2006) and stable isotope techniques (Schneider et al. 2004) have been applied.

Ostracoda are bivalved crustaceans that occur in all types of water bodies, marine, freshwater and (semi-) terrestrial. Because their valves are calcified, they can preserve as microfossils providing an extensive and excellent fossil record (see below). The non-marine ostracods are divided over three major groups, the *Cypridoidea*, *Cytheroidea* and *Darwinuloidea*. The *Cytheroidea* reproduce mainly sexually, the *Cypridoidea* have a high percentage of mixed reproduction, whereas the *Darwinuloidea* are ancient asexuals (Martens 1998). Karyological and allozyme studies have so far only found evidence for apomictic parthenogenetic reproduction in ostracods (summarized in Schön and Martens 2003a).

Darwinulid ostracods are found in all kinds of water bodies and also in (semi-) terrestrial environments. Some species are known from their type-locality only, whereas others have a world-wide distribution (Rossetti and Martens 1998). Studies on the life-history of *Darwinula stevensoni* in Finland (Ranta 1979), Canada (McGregor 1969) and Belgium (Van Doninck et al. 2003a) have shown that, depending on latitude, it can take between 1 and 4 years for this species to complete its life cycle, which is exceptionally long for non-marine ostracods. The type-species of the family, *D. stevensoni*, apparently has developed a general purpose genotype (GPG), which enables it to tolerate a very wide range of salinities and temperatures (Van Doninck et al. 2002). A GPG is one way to overcome the ecological problems associated with ancient asexuality because adaptation to changing abiotic environmental parameters is no longer necessary. Other darwinulid species, however, have limited ecological tolerances or are intermediates between ecological specialists and generalists (Van Doninck et al. 2003b).

Genetic research on *D. stevensoni* started with allozymes (Rossi et al. 1998) and is continuing with DNA-based methods. Intraspecific genetic variability has been estimated for natural populations with several genetic markers from the nuclear and mitochondrial genome (Schön et al. 1998; Schön and Martens 2003b; Schön 2007; Van Doninck et al. 2004b). Some of the most surprising results are the exceptionally low levels of genetic diversity in the screened regions from the nuclear genome (Schön et al. 1998; Schön and

Martens 2003b). These are currently explained by a slow-down in molecular evolution (Martens et al. 2005), highly efficient DNA repair (Schön and Martens 1998) or other homogenizing mechanisms such as gene conversion (Schön and Martens 2003b) (see below for a discussion on the evolutionary consequences). The evolutionary history of the *Darwinulidae* has been reconstructed from phylogenies utilizing both molecular and morphological characters (Martens et al. 2005), which turned out to be congruent. This result follows theoretical expectations as the molecular evolution of different genomic regions is supposed to be linked in ancient asexuals. Recently, genomic-based research on *D. stevensoni* has also been initiated by screening natural populations for transposable elements (Schön and Arkhipova 2006) (see Sect. 2.2.5).

The arbuscular mycorrhizal fungi are supposed to have been asexual for at least 400 myr (Judson and Normark 1996; Sanders 1999). They are widely distributed symbionts having colonized the roots of probably 80% of all terrestrial plant species (Smith and Read 1997). They furthermore account for up to 50% of the total microbial soil biomass (Olsson et al. 1999). Arbuscular mycorrhizal fungi produce asexual spores but these can contain hundreds of genetically different nuclei (Kuhn et al. 2001)⁸.

More extensive screening of fungi populations kept under controlled environmental conditions has revealed that genetic differences between individuals from the same population can be large, whereas total diversity of local populations was low (Koch et al. 2004). Both studies indicate that the multigenomic nature of arbuscular mycorrhizal fungi has important impacts on the genetic and ecological consequences of asexual reproduction. Because of their genetic redundancy, the accumulation of mutations and selection by changing environments might be buffered in these fungi. But how can we be certain that a particular species is indeed an ancient asexual? Below, we will discuss several approaches to provide likelihoods for the long-term absence of sex.

2.1

Classical Non-genetic Methods

2.1.1

Absence of Functional Males in Recent and Fossil Populations

In the early days of research, absence of males was the most obvious evidence for asexuality. If no males had been reported in the fossil record for a long time (e.g. 1 myr), putatively ancient asexuality was considered. Although this makes instant sense, the case is not so simple:

⁸ Hyphal fusion and exchange of nuclei can freely occur within a “compatible” population (Giovannetti et al. 2003). For full documentation of compatible anastomosis in *Glomus*, see Giovannetti et al. (1999).

1. When fossils are available, the record must reveal large enough sample sizes of diagnostically well-preserved specimens
2. Males might only occur at short time periods during the year
3. Males might be tiny, as for example in some monogonont rotifers (Ricci and Melone 1998) and therefore hard to recognize
4. Males may be so different from their female conspecifics that they have been described under other species names (for male structures in fungi, see Wong et al. 2003).

Even if males are found, it is not immediately clear whether they are still functional and can genetically contribute to offspring (Smith et al. 2006). It is not as simple as Little and Hebert (1996) proposed: they predicted that most cases of ancient asexuals would disappear because males had been overlooked. Some putative ancient asexuals had to be removed from the list, such as the Homoptera genera *Trama* and *Neotrama* (Normark et al. 2003), because males have meanwhile been found. However, the search for males is only meaningful if subsequent testing for functionality follows, because *a little sex now and then* (Hurst and Peck 1996) can still be sufficient to overcome the genetic consequences of ancient asexuality.

No bdelloid males have ever been described, while in oribatid mites rare males do occur in some species but they have been shown to be non-functional (Täberly 1987c). In other parthenogenetic mite species, no males have ever been reported (Heethoff, personal communication).

For a long time, no males were known from recent populations of darwinulids, this in spite of extensive sampling efforts, not even from geographic regions where sexual populations are known from ostracods with mixed reproduction, e.g. around the Mediterranean Sea or in ancient lakes (see Martens 1998, for an extensive discussion)⁹. Recently, three males have been found, amongst thousands of females, in a Japanese population of the darwinulid species *Vestalenula cornelia* (Smith et al. 2006). Further research is required to verify whether these are atavistic¹⁰ or functional, and which evolutionary consequences this finding might hold for the ancient asexual status of the whole family.

On the one hand, rare males are not all that uncommon in otherwise all-female populations in other groups of non-marine ostracods, e.g. *Cyprididae*, *Candonidae* and *Limnocytheridae*. For example, Yin et al. (1999) observed two morphological males in cultures of fully parthenogenetic females. Functionality of these males could not be established. The fact that no sperm was found in the three males of *V. cornelia*, nor in any of the sympatric females, can be seen as an indication that these specimens might be non-functional,

⁹ A single putative male of *D. stevensoni* was reported by Brady and Robertson (1889), but the morphology of its so-called hemipenis remained enigmatic and its status was doubted by many (e.g. Rossetti and Martens 1996).

¹⁰ Atavistic: re-expressed from a distant genetic past.

atavistic specimens¹¹. It could also be that only the darwinulid genus *Vestalenula* still has the potential to develop males, whereas this ability has been lost in all other darwinulid species. The fact that up to now no other males have been reported from other darwinulid species supports this point of view¹².

On the other hand, if these males are functional, the incidence of their genetic contribution to offspring needs to be determined. In that case, Darwinulidae as a whole could lose the status of ancient asexuals and only some lineages, e.g. the genera *Darwinula* and *Alicenula*, might remain ancient asexuals.

This example illustrates that male records of putative ancient asexuals should be considered with great care and require close inspection. Their mere occurrence is not sufficient to disprove ancient asexuality. We therefore still discuss the darwinulid ostracods here as a supported case of ancient asexuality.

Confirming *ancient* asexuality requires evidence that sexual reproduction and/or males have been absent for millions of years. There are two ways to estimate evolutionary age of taxa: real time estimates from fossil data and relative time estimates from molecular clocks. Table 1 provides an overview of the known age estimates for the four ancient asexuals and the origin of these estimates. Fossil data are rare for most ancient asexuals except for the *Ostracoda*. If molecular clocks are used instead, the resulting age estimates have to be treated with great care. Hebert et al. (2002), for example, claim that the old age of the putative, ancient asexual shrimp *Artemia parthenogenetica* (Perez et al. 1994) is overestimated because of a speed-up in molecular evolution in 28S. If more appropriate rates of molecular clocks are applied, the longevity of this asexual species shrinks to the time frames of most asexual taxa. The final evaluation on its ancient asexual status will require additional screening of other genomic regions from *A. parthenogenetica* than 28S only. Regardless of whether this species will turn out to be an ancient asexual or not, this example illustrates the need for real time estimates from fossil data instead of applying molecular clocks only.

Bdelloid rotifers are microscopic invertebrates with mainly soft body structures. Fossil data are therefore scarce. The major fossil evidence comes from a single piece of Dominican amber (15–40 myr old), in which 22 specimens of bdelloid rotifers were discovered (Poinar and Ricci 1992). It was not possible to determine genus, species or gender of these specimens. Poinar and Ricci (1992) suggested that this fossil finding could be evidence for ancient

¹¹ It is to the credit of Smith et al. (2006) that they named this species *Vestalenula cornelia*, after the High Vestal Cornelia, the most famous of the Vestal Virgins of the temple of Vesta in Rome. Cornelia was accused of having a secret lover; it is unknown if the accusation was true.

¹² The *Vestalenula* males are morphologically very different from the doubtful male record of *Darwinula stevensoni* (Brady and Robertson 1889) and their description clearly refutes the existence of this older record.

Table 1 Age estimates of ancient asexuals

Group	Taxon	Evidence	Refs.	Age (myr)
Bdelloid rotifers		Fossil amber ^a	Poinar & Ricci (1992)	15–40
Bdelloid rotifers		Molecular clock	Mark Welch & Meselson (2000)	80–100
Oribatid mites	Camisiidae	Fossil amber ^b	Bulanova-Zachvatkina (1974)	85
Oribatid mites	50 genera	Fossil amber ^c	Sellnick (1931) Poinar (1992)	35–40
Oribatid mites	<i>Platynothrus peltifer</i>	Molecular clock	Heethoff et al. (2007)	58–110
Darwinulidae	<i>Alicenula</i>	Fossil assemblage ^d	Martens et al. (2003)	200
	<i>Darwinula stevensoni</i>	Fossil assemblage ^e	Straub (1952)	25
Glomeromycota		Fossil assemblage ^f	Redecker et al. (2000)	465
		Fossil assemblage ^g	Remy et al. (1994)	400
		Molecular clock	Heckman et al. (2001)	1200–1400

^a Dominican amber^b Cretaceous amber from the Russian Kara Sea^c Baltic amber^d Jurassic limestone, Purbeck deposit, Southern UK^e Tertiary limestone, Oberschwaben, Southern Germany^f Ordovician dolomite rock, Wisconsin, USA^g Devonian rock, Rhynie Chert, Scotland

asexuality if the bdelloids in the amber had the same reproductive mode as today. Additional proof for this hypothesis is still lacking. By applying molecular clocks to sequence data from different nuclear genes, Mark Welch and Meselson (2000) estimated that bdelloids might have been asexual for about 80 myr, maybe up to 100 myr (Table 1).

The *Oribatida* are heavily chitinized arthropods facilitating fossilization. From the Gilboa deposit, they are known since the Devonian, thus, the group is at least 380 myr old (Shear et al. 1984; Norton et al. 1988). Species from the exclusively parthenogenetic group Camisiidae have been found in northern Russian Kara Sea amber from the the Cretaceous (Bulanova-Zachvatkina 1974) with an age of 85 myr. Fifty genera of oribatid mites were reported from Baltic amber (35–40 myr) (Sellnick 1931; Poinar 1992). Using COI sequence data and molecular clocks, Heethoff et al. (2006) estimated the age of

the parthenogenetic species *Platynothrus pelifer* to be at least 15–58 myr and possibly up to 110 myr.

Fossil records of ostracods are without doubt the most extensive and reliable when compared to the other groups of putative ancient asexuals. With one exception, all darwinulid species are brooders, causing a valve dimorphism that can be used to check fossil populations for the absence or presence of males. The fossil record strongly supports that darwinulid ostracods originate from sexual ancestors. About 250 myr ago, during the Permian–Triassic boundary, most darwinulid lineages (> 200 species) became extinct; apparently, only a single fully parthenogenetic lineage survived. At present, the family consists of about 30 species in five genera (Rossetti and Martens 1998). Martens et al. (2003) have extensively re-screened fossil assemblages of one species from the darwinulid genus *Alicenula* to provide more precise estimates on when sexual reproduction stopped. It turned out that parthenogenesis in this genus might have existed for as long as 200 myr. For the type species of the family, *Darwinula stevensoni*, Straub (1952) found fully parthenogenetic populations as far back as 25 myr.

The fossil record of the fungi phylum *Glomeromycota*, to which the arbuscular mycorrhizal fungi belong, goes back to the Ordovician (Redecker et al. 2000), about 460 myr ago. Age estimates based on molecular clocks date the origin of *Glomeromycota* back to 1200 or 1400 myr ago (Heckman et al. 2001). Whether sexual reproduction has indeed been absent for the whole period since this phylum originated, is not clear (Pawlowska 2005).

2.2

Classical Genetic Techniques

If the absence of males in recent and/or fossil populations is not the best proof for ancient asexuality, other, more genetically derived tests might be more appropriate. The accumulation of mutations (Kondrashov 1993; Muller 1964) is still one of the most accepted theoretical consequences for long-term asexuality. We will refrain from discussing the relevance of these hypothesis here (see chapter by Lankenau, in this BOOK), but will rather point out how the theoretical predictions can be used to test for an ancient asexual status.

2.2.1

Population Genetic Data

If sexual reproduction and, consequently, recombination are absent, this will become visible in population genetic analyses such as tests for Hardy–Weinberg equilibrium, linkage equilibrium etc. Given that the appropriate molecular tools are available, these kinds of tests can be and have been applied to a wide range of organisms. They are probably not applicable to arbuscular mycorrhizal fungi, however, because of their multigenomic na-

ture (Kuhn et al. 2001). Although data from population genetics are useful for defining whether asexual reproduction is apo- or automictic (see Schön and Martens (2003a) for a review on ostracods and Gómez (2005) for a review on rotifers), it is possible that sex and/or recombination are so rare that they remain undetectable with this kind of screening. In these cases, other genetic or molecular tests have to be used (see below).

In contrast to most plants and animals, the majority of eukaryotic microorganisms can reproduce asexually (Burt et al. 1996) although very often, cryptic sex became obvious when the species in question were studied more extensively. Population genetic data have shown that the human pathogens *Coccidioides immitis* and *Histoplasma capsulatum*, for example, undergo frequent recombination events (Burt et al. 1996; Carter et al. 1996).

2.2.2

The Meselson Effect

For the debate on ancient asexuality, the predicted outcome of Kondrashov's mutation accumulation theory (Kondrashov 1988, 1993) and Muller's ratchet (Muller 1964) are of great importance: if meiosis and recombination have been absent for millions of years, not only the genome as such but also the two (or more) alleles of any homologue loci must have become degenerated by the accumulation of mutations. This prediction was firstly put forward by White (1973) and has meanwhile been republished by Birky (1996) and Judson and Normark (1996). It is now commonly known as the "Meselson effect" and should provide a clear-cut test for ancient asexuality by comparing genetic variability of single-copy genes within and between individuals.

The Meselson test has meanwhile been applied to representatives from all four ancient asexuals: four species of bdelloid rotifers (Mark Welch and Meselson 2000), the ostracod species *Darwinula stevensoni* (Schön and Martens 2003b), several oribatid mite species (Schäfer et al. 2006) and several species of arbuscular mycorrhizal fungi (Hijri and Sanders 2005; Kuhn et al. 2001; Pawlowska and Taylor 2004). Also, placozoans occupying a basal position at the metazoan tree were screened as an additional putative ancient asexual group (Signorovitch et al. 2005) but ancient asexuality had to be refuted (see below).

For representatives of the three ancient asexuals from the animal kingdom, several nuclear genes such as *EF1 alpha*, *tbp*, *rpol3I*, *tpi* and *hsp82* have been analysed. Also, the multi-copy region ITS and a Calmodulin intron were investigated for *Darwinula stevensoni* (Schön and Martens 2003b). However, *hsp82* is the only nuclear region that has been investigated for representatives of all three groups and can be used to directly compare results. If HKY85 (Hasegawa et al. 1985) is used to estimate genetic distances, the maximum diversity observed within an individual bdelloid rotifer is 0.1516, ten times higher than in an oribatid mite (0.012) and a darwinulid ostracod (0.015).

It is obvious that only the bdelloid rotifer species apparently (see further) shows evidence for the Meselson effect, whereas the intra-individual, genetic diversity remains low for representatives of the other two taxa. As a matter of fact, it equals the genetic distance between individuals and, in the case of *D. stevensoni*, even between geographically different populations. Thus, only the screened bdelloids show the expected high genetic divergence between the different alleles of *hsp82*, with up to 54% divergence for fourfold degenerate sites (Mark Welch and Meselson 2000¹³). Phylogenetic analyses further revealed that the different copies formed statistically supported clusters in trees that followed genetic and not species groupings (Butlin 2002) as theory had predicted (Birky 1996).

In the case of arbuscular mycorrhizal fungi, several papers report high degrees of genetic divergence between ribosomal sequences of individual Glomales (Hijri et al. 1999; Hosny et al. 1999), which was partly due to contamination with other fungal species (Redecker et al. 1999). However, the high levels of genetic differentiation within individual spores could be reconfirmed by FISH analysis of rDNA (Kuhn et al. 2001) and additional analyses of Pol-like sequences (Hijri and Sanders 2005; Pawlowska and Taylor 2004). This finding cannot be explained by polyploidy because all species that were meanwhile screened have small genome sizes and are most likely haploid (Bianciotto and Bonfante 1992; Hijri and Sanders 2004; Hosny et al. 1998).

Signovoritch et al. (2005) applied a similar test to the *Placozoa*. Instead of amplifying specific nuclear genes, however, random clones were sequenced from cDNA and the genetic identity was afterwards verified with TBLASTX search. Although the results are not directly comparable with the ancient asexuals mentioned above, because other nuclear regions were sequenced, the absence of the Meselson effect is also obvious in the *Placozoa*. None of the seven sequenced genes showed the expected increased levels of genetic diversity within as compared to between individuals (Signovoritch et al. 2005). Several individuals showed a mix of alleles that can only be explained by meiotic recombination. Thus, for the *Placozoa*, the Meselson effect proved to be appropriate for testing and rejecting ancient asexuality.

2.2.3

Asymmetry and Decline of the Meselson Effect

At first glance, it seems that the studies above confirm the ancient asexuality status for one group only, the bdelloid rotifers. However, for several bdelloid species, Mark Welch and Meselson (2000) found more than the two alleles that would be expected from an ancient asexual diploid. For *Philodina roseola*, they found four different copies of *hsp82* and for *Habrotrocha constricta* and *Adineta vaga* three. Since there is no evidence from FISH that *hsp82*

¹³ This may, however, be owing to an ancient hybridization event, see Sect. 2.2.3.

has become a multi-copy gene in bdelloids, ancient hybridization is the most likely explanation (J. Mark Welch et al. 2004). This would mean that bdelloid rotifers are allotetraploids and that the observed genetic differences within individual bdelloids are actually the genetic distances between the ancestral genomes. Whether the different copies started to diverge before or after the bdelloid radiation, cannot be discriminated from gene trees constructed with available DNA sequence data (Mark Welch et al. 2004). Alternatively, (Omilian et al. 2006) suggested that mosaic patterns of allelic diversity being generated by ameiotic recombination could provide an explanation for the observed diversity within bdelloid rotifers. Thus, even the only positive example of the Meselson effect might not hold true.

As Butlin (2000) pointed out, there is also asymmetry connected with the Meselson effect. Its presence confirms ancient asexuality but its absence can be owing to factors other than the presence of meiotic recombination. It is commonly agreed that homogenizing mechanisms such as gene conversion and DNA repair take place mitotically (Butlin 2002; Omilian et al. 2006; Haber, in this BOOK). In this case, these mechanisms can erase any evidence for ancient asexuality and, consequently, also for the Meselson effect. The most parsimonious explanation for the absence of the Meselson effect in oribatid mites is their automictic reproduction (Schäfer et al. 2006). For *Darwinula stevensoni*, the explanation is probably less simple. Likelihood permutation tests could provide evidence for gene conversion in the multi-copy region ITS but not in the single-copy, nuclear *hsp82* gene (Schön and Martens 2003b; see also below). Other explanations such as highly efficient DNA repair (Schön and Martens 1998) might therefore be more appropriate.

Finally, additional doubt has been raised on whether the Meselson effect is indeed a suitable test for ancient asexuality. Ceplitis (2003) modelled coalescent times¹⁴ for pairs of gene copies in asexual populations by using a slightly modified version of the commonly used two-deme population structure. He shows that the high genetic divergences within individual bdelloid rotifers, as estimated by Mark Welch and Meselson (2000), are compatible with one instance of sexual reproduction every seven generations. This would imply that even positive evidence for the Meselson effect is no longer sound proof for ancient asexuality.

2.2.4

Countering Mechanisms Against Mutation Accumulation

Although mitotic crossing-over is a hundred to a thousand times less frequent than meiotic recombination (van Heemst and Heyting 2000), it might have important genetic consequences in the long-term absence of sex. If mitotic homogenizing mechanisms such as the ancient synthesis-dependent strand an-

¹⁴ In “coalescent theory”, this is the time elapsed from the most recent common ancestor.

nealing (SDSA) mechanism (Lankenau 2007) can indeed counter the expected accumulation of mutations in ancient asexuals, testing their presence is highly important for the whole debate on ancient asexuality. If genetic divergence between alleles is sufficiently high, as in the case of the *Placozoa*, simple analyses on how these alleles are distributed can already provide evidence for (meiotic) recombination (Signorovitch et al. 2005). However, the genetic patterns are rather different for most ancient asexuals. Assuming that these homogenizing mechanisms have been active for millions of years, only single nucleotides will differ between alleles, making statistical analyses difficult.

The choice of the suitable statistical method to test for recombination with reciprocal exchange seems the most important prerequisite to obtain trustworthy evidence for recombination (Posada 2002; Carvajal-Rodriguez et al. 2006). More than 15 different, statistical tests for recombination and gene conversion have been evaluated by Posada and Crandall (2001), Posada (2002) and Carvajal-Rodriguez et al. (2006) from both simulated and empirical data of sexual taxa. These tests are mainly based on phylogenetics or population genetics and provide evidence for the absence or presence of recombination (see Table 2 for an overview).

Table 2 Overview on statistical methods and computer packages to check for recombination and gene conversion

Name/method	Category	Performance ^a	Refs.
Geneconv	Substitution	++	Sawyer (1999)
Homoplasy test	Substitution	–	Maynard Smith & Smith (1998)
Simplot	Phylogenetic	–	Salminen et al. (1996) Lole et al. (1999)
Pist	Substitution	+	Worobey (2001)
MaxChi2	Substitution	++	Maynard Smith (1992)
Chimaera	Substitution	+	Posada & Crandall (2001)
Phypro	Distance	–	Weiller (1998)
Plato	Phylogenetic	–	Grassly & Holmes (1997)
Rdp	Phylogenetic	+	Martin & Rybicki (2000)
Recpars	Phylogenetic	–	Hein (1990)
Reticulate	Compatibility	++	Jakobsen & Easteal (1996)
Runs test	Substitution	–	Takahata (1994)
Sneath test	Substitution	++	Sneath (1995)
Triple	Phylogenetic	+	Kuhner et al. (1991)
Likelihood permutation	Several parameters for likelihood model	^b , ^c	McVean et al. (2002)

^a Performance according to Posada & Crandall (2001) and Posada (2002)

^b Especially useful for low rates of recombination and gene divergence

^c Test for gene conversion

The McVean test¹⁵ seems particularly suitable for ancient asexuals because it has the greatest statistical power of all verified methods, even when rates of recombination and genetic divergence are low, features that would be expected in ancient asexuals. Only two papers have so far applied this test by McVean et al. (2002) to ancient asexuals. Schön and Martens (2003b) did not find any statistical evidence for recombination in *hsp82* or an intron of the Calmodulin gene in the putative, ancient asexual *D. stenosoni*; they observed only statistical evidence for gene conversion in the ribosomal ITS1 region. Schäfer et al. (2006) did not find evidence for gene conversion in either *hsp82* or *EF1 alpha* from several oribatid mite species.

It is obvious from the above that applying appropriate statistical techniques to DNA sequence data from putative ancient asexuals to test for recombination can be a suitable strategy to verify or reject ancient asexuality. New statistical techniques are still being developed (see for example Kosakovsky Pond et al. 2006) and one can hope that more extensive sequence data from ancient asexuals will confirm the patterns observed so far.

Tests for gene conversion are far less common (see Table 2). Their statistical power with simulated and empirical data has not yet been assessed. This is a great pity because, from the (apparent) failure of the Meselson test (see above) in most ancient asexuals screened so far, it seems obvious that gene conversion and other pathways with comparable features might not only be much more common in non-recombining systems than previously thought (see, for example, the human Y chromosome; Rozen et al. 2003), but they might also be of essential importance for most, if not all, ancient asexuals to keep their genomes free from the expected accumulation of mutations.

Even with statistical tests it is difficult to assess how long in evolutionary terms signals for recombination might endure in ancient asexuals. Gandolfi et al. (2003) could apparently show that all three analysed groups of ancient asexuals retained recombination¹⁶. That the ancestors of bdelloid rotifers and arbuscular mycorrhizal fungi had sex and also recombination, is unquestioned. What we do not know at the moment, however, is when meiotic recombination aside from DSB repair was abandoned.

The frequency and evolutionary importance of recombination in arbuscular mycorrhizal fungi is still not conclusively answered. Population genetic-based studies on these fungi have provided evidence for both recombination (Vandenkoornhuyse et al. 2001) and clonality (Rosendahl and Taylor 1997; Stukenbrock and Rosendahl 2005). Further evidence for recombination could be found in rDNA of arbuscular mycorrhizal fungi but not in the BiP gene from the same species (Kuhn et al. 2001). Because of their multinuclei and multigenomic nature, even rare events of recombination might not be

¹⁵ A composite-likelihood estimator for the recombination rate being an extension of the method by Hudson (2001).

¹⁶ A significance level of 0.56 in *D. stenosoni* was seen as statistically significant evidence for recombination.

sufficient to purge the majority of deleterious mutations from these fungi genomes (Kuhn et al. 2001). This hypothesis will require additional investigations before it can be irrefutably concluded whether arbuscular mycorrhizal fungi are ancient asexuals or not.

2.2.5

Presence/Absence of Transposable Elements

Hickey (1982) predicted that transposable elements would spread in populations even if they introduce deleterious mutations into their hosts, under one important prerequisite: that the reproductive mode is sexual. Otherwise, these elements are locked into individual genomes without the possibility to invade new genomes. This prediction has meanwhile been re-discussed by others (Arkhipova and Meselson 2005a; Schön and Martens 2000; Wright and Finnegan 2000). Experimental studies on yeast could indeed prove that the retrotransposon Ty3, which had been actively introduced into populations with different reproductive modes, had a higher probability to spread and a higher abundance in sexual as compared to asexual populations (Zeyl et al. 1996). Arkhipova and Meselson (2000) could find further support for this pattern through an extensive screening of 46 animal species belonging to 26 phyla. They used degenerated PCR primers for the reverse transcriptase domain of retrotransposons and the transposase of DNA transposons, respectively. The most consistent pattern was the absence of both LINE- and gypsy-like retrotransposons from the five ancient asexual bdelloid species that were screened. Some of the closely related *Monogononta* with mixed reproduction, in contrast, had LINE- and gypsy-like retrotransposons. Bdelloid rotifers were found to have mariner-like but no Tc1-like DNA transposons. More extensive research on the bdelloids has meanwhile revealed that there is a single retroelement named *Athena*, which has most likely become domesticated for telomeric functions (Arkhipova et al. 2003). Arkhipova and Meselson (2005b) found representatives of at least five superfamilies of DNA transposons in bdelloid rotifers, of which mariner-like elements were most numerous and exhibited evidence of recent activity. Also, some copies of the ITm superfamily were probably still intact. This is not in contradiction with the theoretical predictions mentioned above because it is known from other taxa that mariner- and ITm-like elements are horizontally transmitted, the former even between pro- and eukaryotes. Most representatives of other DNA transposons were present in low copy numbers and found to be deteriorated in bdelloid rotifers. Interestingly, they were more often found in telomeric regions associated with the retroelement *Athena* than in gene-rich regions.

Other studies on putative ancient asexuals provide comparable, albeit less clear patterns. For the diplomonad *Giardia intestinalis* (*syn. lamblia*), no sexual cycle has ever been observed (Adam 2001). Because this protist is

a representative of an old eukaryotic lineage (Baldauf 2003), it could have evolved before meiosis and sex made their way into eukaryotic evolution. However (as discussed further below), genes encoding a core set of meiosis functions have been detected in the *Giardia* genome (Ramesh et al. 2005). This is indicative of a yet older origin of meiosis (see Egel and Penny, in this BOOK). Arkhipova and Morrison (2001) identified three families of LINE-like retrotransposons in *Giardia lamblia*, of which one had lost functionality and two were restricted to telomeric regions. Although they probably did not replace telomeric functions as in *Drosophila*, their preference to telomeric and subtelomeric regions might indicate that they could function as an additional buffer in *Giardia* between chromosome ends and telomeres.

Schön and Arkhipova (2006) screened the ancient asexual ostracod species *Darwinula stevensoni* for LINE-like elements, of which they found two different families. One of them, *Daphne* not only shows the expected accumulation of mutations but in addition several structural abnormalities such as loops and inversions. Furthermore, from the ratio of synonymous to non-synonymous substitutions, the authors could conclude that there is no evidence for recent activity. The second family named *Syrinx*, however, is still present in the genome of *D. stevensoni* with some intact copies and has recently been active (as derived from the ratio of synonymous to non-synonymous substitutions). Results from simulation studies (Docking et al. 2006; Dolgin and Charlesworth 2006) indicate that ancient asexuals might be unable to purge their genomes from retroelements under certain circumstances (see below). Modelling the evolution of retroelements together with the life-history parameters and mutation rate of *D. stevensoni* is required before it will be obvious whether the exceptionally low mutation rate of *D. stevensoni* (Schön and Martens 2003), efficient DNA repair (Schön and Martens 1998) or other factors might be responsible. An alternative explanation could be that *Syrinx* elements became domesticated in *D. stevensoni*, possibly also for telomeric function. The UTR 3' repeats of *Syrinx* are very unusual structural particularities for gypsy-like retroelements, which normally lack such repeats. The 3'UTR have, however, been found in retrotransposons with telomeric functions in *Drosophila* (Pardue and DeBaryshe 2003). This alternative hypothesis will require additional genomic research before it can be confirmed.

The genome of *Candida albicans* contains representatives of 34 related families of long-terminal-repeat (LTR)-retrotransposons (Goodwin and Poulter 2000). They are unique to *C. albicans* and are phylogenetically most closely related to the various Ty families from common yeast. Most of them occur in low copy numbers and are deteriorated, with only two to three copies per family remaining functional (Goodwin and Poulter 2000). The most obvious explanation is that the remnants of ancient elements have not been sufficiently purged from the genome. On the contrary, new elements constantly appear, which in turn become non-functional (Goodwin and Poulter 2000). This pattern is very different to that in yeast, where only five different fam-

ilies of retrotransposons have been found with high numbers of functional copies. Whether this is mainly owing to different incidences of sexual reproduction or differences in effective population size (Dolgin and Charlesworth 2006) is not certain. It could also be explained by other factors such as differences between in-vitro cultures and fungi in the field, the non-standard genetic code of *C. albicans* or constitutive stress (Goodwin and Poulter 2000). Goodwin and Poulter (2000) further suggest that large-scale chromosomal rearrangements in *C. albicans* could have been caused by transposable elements hampering chromosome pairing during meiosis. This could be an interesting evolutionary avenue into asexual reproduction.

Five asexual plant species were screened for transposable elements by Docking et al. (2006). The selected species represent a continuum from recent incidence of asexuality (*Taraxacum* and *Hieracium*) to the older asexual fern, *Vittaria lineate*. All plant species contained Ty1/*copia* and Ty3/*gypsy* elements. The majority of copies obtained for Ty1/*copia* family were intact whereas most copies of the Ty3/*gypsy* elements contained stop codons. LINE-like elements were only characterized from *Taraxacum*. Thus, it appears that all asexual plant species investigated contained vertically transmitted retrotransposons. The authors provide several explanations. Because PCR-based techniques were used, intact copies might be over-represented as they would more efficiently amplify. It is not unlikely, however, that all chosen plant species are of recent asexual origin. Docking et al. (2006) did not exclude the possibility of horizontal transfer, possibly between sexual and asexual populations. At least to our knowledge, this kind of research has not yet been conducted but is necessary because most of our knowledge on transfer modes of transposable elements comes from sexually reproducing taxa such as *Drosophila*.

Two studies used computer simulations (Dolgin and Charlesworth 2006, Docking et al. 2006) to predict the fate of retroelements in long-term asexuals. Docking et al. (2006) showed that active elements¹⁷ will eventually get lost but that this process can take ten thousands of generations and will depend on the transposition rate, the overall mutation rate, the strength of purifying selection and the possibility of excision. If all of these parameters are at the “worst” scenario, stable numbers of retrotransposons are never reached during simulations. Retroelements also do not disappear from genomes of ancient asexuals if they are not regularly inactivated by mutations. This could be relevant for the case of *Darwinula stevensoni*, where LINE-like, active elements were observed (Schön and Arkhipova 2006; see above). Interestingly, the modelling of Docking et al. (2006) reveals an initial increase of the number of transposable elements prior to the transition to asexuality, which can reach more than 100 copies per individual.

Dolgin and Charlesworth (2006) demonstrate that population size is most crucial for determining the fate of retroelements in long-term asexuals, a factor

¹⁷ Active elements are indicated by the ratio of non-synonymous to synonymous mutations.

that was not considered by Docking et al. (2006). In large (infinite) populations an equilibrium in the number of TE is achieved in those simulations without elements being excised. In large populations with the possibility of excision, all elements can be purged from the asexual population. Such populations will be immune against further transposition if there is no horizontal transfer (as in most DNA transposons). The scenario of an infinite population with excision might reflect the patterns in bdelloid rotifers quite well (see above)¹⁸, where no retrotransposons but several DNA transposons have been found.

Dolgin and Charlesworth (2006) also found that small (finite) populations of ancient asexuals are more prone to retrotransposons: without the possibility to excise retroelements, a substantial increase in numbers of TE takes place. Even with excision, deleterious TE will accumulate in small populations. According to Dolgin and Charlesworth (2006), there are two additional factors affecting the proliferation of TE: the transposition-excision rate and the synergism between elements. Both a lower transposition-excision rate and an increased synergism reduce the population size. These are required for the elimination of TE because an increased synergism strengthens selection against TE and lower rates of transposition slow down the rate of accumulation. This implies that TE are effectively removed from large populations regardless of the initial copy number, whereas slight differences in the two parameters can shift the balance between selection and genetic drift, resulting in either accumulation or purging of TE. If most TE are not deleterious but have inserted in neutral sites, their elimination in asexual populations will be much slower (Dolgin and Charlesworth 2006). From the simulations by Dolgin and Charlesworth (2006), several explanations can be put forward for the discovery of active LINE-like *Syrinx* elements in *Darwinula stevensoni* (Schön and Arkhipova 2006; see also above). This could either be owing to a large initial copy number, a small population size, a low excision or high transposition rates, the lack of synergism between elements or the fact that most TE have inserted into neutral sites.

In conclusion, the screening for functional transposable elements with vertical transposition can be indicative for ancient asexuality. Characterizing retrotransposons is promising because they are most prone to changes in copy numbers due to their replicative mode of transposition. However, the results described above show that even from theoretical considerations, clear-cut patterns are rare and the balance between accumulation and the purging of TE is delicate. It can be shifted either way by small changes in parameters such as population size, synergism between TE, excision and transposition rates (Dolgin and Charlesworth 2006).

There are a few other arguments that might make it less simple to use the presence of active retroelements as evidence against ancient asexual-

¹⁸ This is not supported by the fact that most retrotransposons are probably not excised (Craig et al. 2002).

ity. Firstly, transposable elements might not be as deleterious as originally believed. They are known to insert also into neutral sites (Charlesworth 1991) and there is evidence for instances of “domestication”, when transposable elements acquire beneficial functions for the host. The involvement with (sub)telomeric functions seems rather likely from the examples provided above. Secondly, most of our knowledge on transposition modes and rates and on excision rates or other means of inactivation comes from a few sexually reproducing species. The picture might be very different in ancient asexuals, which is why additional studies cannot be encouraged enough. Thirdly, the strong association between the loss of functionality in transposable elements and the mutation rate of the host might have important implications for ancient asexuals. If these either have developed homogenizing mechanisms (see above) or have obtained especially low mutation rates, they might never be able to prune their genomes from all retroelements or this process might still be going on, even after millions of years. Last, but not least, transposable elements could provoke large chromosomal rearrangements that would hamper both meiotic pairing and sexual reproduction. In the latter case, transposable elements could be the cause of (ancient) asexuality in some taxa.

2.2.6

Aneuploidy

Odd chromosome numbers and morphological uniqueness have been considered as indication for reproduction without meiosis (Judson and Normark 1996). Studies on the chromosomal structure of asexuals could therefore provide additional evidence for ancient asexuality. In some sexual species, however, single chromosomes can pair with two partners in meiosis (White 1973). Whether this could also be the case in putative ancient asexuals is not known.

So far, there is only evidence from a single species of ancient asexuals, namely the bdelloid rotifer *Philodina roseola*, for having odd chromosome numbers (J. Mark Welch et al. 2004). The other three screened bdelloid species have chromosomes that cannot be distinguished by their morphology (J. Mark Welch et al. 2004). Likewise, *Darwinula stevensoni* has 22 dot-like chromosomes where homologue pairs cannot be identified (Tétart 1978). Also the oribatid mite species *Archegozetes longisetosus* shows small chromosomes that could not be further differentiated (Heethoff et al. (2006)).¹⁹

Additional karyological studies on more representatives from all three ancient asexual animal groups will hopefully allow further verification.

¹⁹ Whether oribatid mites indeed show inverted meiosis, as has previously been suggested (Wrensch et al. 1994) remains to be confirmed (Heethoff et al. 2006).

3

Novel Genetic Tests – Meiosis Proteins

An alternative (genetic) test for ancient asexuality involves screening for functional meiosis proteins. Table 3 provides an overview of possible candidate genes. If meiotic recombination has been absent for millions of years, the proteins in question should have become deteriorated through the accumulation of mutations. Or, in the case that they are still functional, they have acquired new functions such as the vertebrate *DAZ* (*deleted in azoospermia*) genes that originated from the single-copy gene *Boule* (Xu et al. 2001).

If presence and functionality of meiosis proteins are used to test for ancient asexuality, several practical considerations have to be taken into account. Firstly, it would be expected that the absence of certain meiosis proteins in ancient asexuals is owing to sequence degradation. Detection by PCR amplification or FISH of BAC libraries might become impossible because primers or probes based on conserved sequences would no longer bind. How fast functionality of a certain protein is lost in the natural world is often not known and so far mostly reported in anecdotic papers. Fryer (1999), for example, postulated that the master gene for eye development must have remained functional in Anostraca for 300 myr or more. Molecular proof for this hypothesis has not been provided. To make matters worse, some putative ancient asexuals such as darwinulid ostracods might have much slower net mutation rates than sexual relatives (Schön et al. 2003). This would also imply that the corruption of meiosis genes might take millions of years.

Secondly, the experimental design has to rely on conserved proteins, for which sequence data from model organisms such as *Caenorhabditis elegans*, *Drosophila melanogaster* or *Saccharomyces cerevisiae* are available. This can become problematic if the organism in question is phylogenetically too distant from such model organisms. However, a similar approach has been successful for screening of the protist *Giardia* (see below).

Thirdly, not all conserved eukaryotic proteins are present in all eukaryotes. Ramesh et al. (2005) observed a rather patchy distribution where certain meiotic genes have obviously been lost in eukaryotic model taxa. The plant species *Arabidopsis* and *Oryza*, for example, lack *Rad52* and *Mlh2*, whereas six meiotic proteins (*Hop2*, *Mnd1*, *Rad52*, *Dmc1*, *Mlh2* and *Mlh3*) are not known from the invertebrate model species *Caenorhabditis*, *Drosophila* and *Anopheles*. If putative ancient asexuals are screened for similar sets of proteins, it might not be conclusive whether the absence of certain proteins is linked to asexual reproduction or other unknown factors of molecular evolution.

Fourthly, and most importantly, the majority of meiotic proteins have more than just meiotic functions. Table 3 demonstrates that only seven out of 17 proteins are expressed for purely meiotic functions. Since this conclusion

Table 3 Overview on meiotic proteins from eukaryotes. Modified according to Ramesh et al. (2005)

Meiotic protein	Function	Taxon	Refs.	<i>Giardia</i>
Boule	Meiotic regulator in males	<i>Drosophila</i>	Eberhardt et al. (1996)	?
Boule	Meiotic regulator during oogenesis	<i>C. elegans</i>	Karashima et al. (2000)	?
Dmc1	Meiotic recombination	<i>S. cerevisiae</i> <i>Arabidopsis</i>	Masson & West (2001) Shinohara et al. (1997) Couteau et al. (1999) Klimuyuk & Jones (1997)	+
Hop1	Functions during meiotic prophase I	<i>S. cerevisiae</i>	Hollingsworth et al. (1990) Kironmai et al. (1998)	+
Hop2	Functions during meiotic prophase I	<i>S. cerevisiae</i>	Leu et al. (1998) Tsubouchi & Roeder (2002)	+
Mlh1–3	Mismatch repair, meiotic crossover	<i>S. cerevisiae</i>	Hunter & Borts (1997) Hoffman et al. (2003) Wang et al. (1999)	+
Mnd1	Meiotic recombination	Eukaryotes	Tsubouchi & Roeder (2002) Zierhut et al. (2004) Gerton & DeRisi (2002)	+
Mre11	DS DNA exonuclease, SS DNA endonuclease;	Eukaryotes	Haber (1998) Moreau et al. (1999)	+
Msh2, Msh6	Mismatch repair	<i>S. cerevisiae</i>	Reenan & Kolodner (1992)	+
Msh4 Msh5	Meiotic recombination	<i>S. cerevisiae</i>	Pochart et al. (1997) Novak et al. (2001)	–
Pms1	DNA repair of heteroduplex DNA	Eukaryotes	Borts et al. (2000)	+
Rad50	DNA-binding protein for broken DNA ends	Eukaryotes	Connelly & Leach (2002)	+
Rad51	Mitotic recombination, DNA damage repair	<i>S. cerevisiae</i>	Shinohara et al. (1992, 1997)	–
Rad52	Binds DBS	Eukaryotes	Gasior et al. (1998)	+
Spo11	Creates DBS during meiosis	<i>S. cerevisiae</i> <i>Arabidopsis</i>	Keeney et al. (1997) Grelon et al. (2001)	+

Proteins printed in bold are those that have meiosis-specific functions in the investigated taxa. Functions are only briefly described; interested readers should refer to the respective chapter of this volume or the provided references. *Giardia* refers to the screening by Ramesh et al. (2005)

DS double strand, SS single strand, *S. Saccharomyces*, ? not tested

is based on data from a rather limited set of model organisms, the real number might be even lower in ancient asexuals. One could easily imagine that in the absence of meiosis, *Spo11* could have acquired novel functions for introducing DSB during DNA repair in *Darwinula stevensoni* (Schön and Martens 1998) or during desiccation in bdelloid rotifers (M. Meselson, personal communication). If genes linking meiosis and DNA repair, as for example *Rad51*, *Mlh1-3* or *Pms1*, would be functional in ancient asexuals, this could either be explained by the presence of meiosis or by highly efficient DNA repair (Schön and Martens 1998) and could not provide a clear-cut test. In ancient asexuals with automixis, meiosis genes would be expected to remain functional.

The meiosis-gene based test for ancient asexuality has so far only been conducted twice. Ramesh et al. (2005) screened *Giardia* for 17 highly conserved meiotic proteins by a PCR-based approach²⁰. Surprisingly, 15 out of the 17 meiotic proteins appeared to be present in *Giardia* (see last column of Table 3) including the seven proteins with purely meiotic function. Further sequence analyses revealed that none of these meiotic proteins appeared to be corrupted by mutations. Whether these proteins still function in vivo will have to be verified with additional gene expression experiments. In any case, if one rejects the unlikely scenario that seven meiotic genes acquired novel functions in *Giardia*, the most parsimonious explanation is that *Giardia* has some form of cryptic, yet unobserved sex (Birky 2005).

Genomic screening of the human pathogenic yeast, *Candida glabrata*, identified 19 orthologues of *S. cerevisiae* genes being exclusively involved in meiosis or sporulation (Wong et al. 2003). These orthologues include the master regulatory switch gene IME1, with its inductors (MCK1 and RIM9) and regulator (UME6), and IME2 (which induces sporulation in the absence of IME1) together with its regulators IDS2 and RIM4. Orthologues to other early meiosis proteins such as MUM2, HOP2 and MSH4 could also be identified. Similarly, Wong et al. (2003) found orthologues of genes being involved in middle and late meiotic stages in *S. cerevisiae* such as SPO1 and SPO22, CSM1 and CSM3, SMK1 and DIT1. The conclusion from this genomic screening is clear: *C. glabrata* undergoes sexual cycles that have so far remained undiscovered during almost 100 years of scientific research, or it lost sex relatively recently.

4

Conclusions

Although others might disagree, we feel that we have presented sufficient evidence for ancient asexuality of the bdelloid rotifers, darwinulid ostracods

²⁰ For the design of primers, published protein sequences were mined for meiotic protein homologue(s), which were in turn used to query the *Giardia* database.

and oribatid mites. What are the mechanisms that allow these taxa to survive millions of years without sex and meiosis?

Large population sizes are an important factor to overcome two potential problems of long-term asexuality: Muller's ratchet (e.g. Gordo and Charlesworth 2000) and the accumulation of retrotransposons (Dolgin and Charlesworth 2006). Although estimates for effective population sizes of any of the three groups are not yet available, their small size and high density²¹ hint towards large population sizes. Living in stable environments as oribatid mites and darwinulid ostracods do, could further sustain large populations.

Homogenizing mechanisms through highly efficient DNA repair (Schön and Martens 1998), ameiotic recombination and gene conversion (Omilian et al. 2006) might provide additional mechanisms to overcome the expected accumulation of mutations. The fact that the Meselson effect could not be confirmed for any of the ancient asexuals indicates that such mechanisms might be more common than previously thought. Omilian et al. (2006) found that loss of heterozygosity through ameiotic recombination in obligate asexual lineages of *Daphnia* happened a thousand times faster than the production of heterozygosity through mutations. If ameiotic recombination occurs at least as frequently in ancient asexuals, they could persist without the expected accumulation of mutations. Because frequency and outcome of ameiotic recombination will depend on chromosome location, ameiotic recombination will produce genetically variable offspring being mosaics of their parents (Omilian et al. 2006). Offspring will have both higher and lower fitness than their parents, slowing down any selection-based processes that could eventually lead to the extinction of ancient asexuals.

Other factors contributing to their survival are probably more specific for each of the three groups of ancient asexual animals. Bdelloid rotifers might regularly suffer increased DSBs during desiccation, which in turn could facilitate ectopic genetic exchange (M. Meselson, personal communication). This hypothesis is currently being further tested.

Oribatid mites might have automictic recombination with terminal fusion, which would normally lead to homozygosity. In the case of inverted meiosis and parthenogenetic reproduction, however, this would conserve maternal genotypes (Hetthoff et al. 2006). Karyological studies are currently under way (M. Heethoff, personal communication) to clarify which type of meiosis occurs in asexual oribatid mites.

The fossil record shows clearly that darwinulid ostracods were once far more speciose, while they reproduced sexually (Martens et al. 1998). The currently living representatives of ancient asexuals can thus be regarded as the only survivors of a mass extinction. We can only speculate whether these "lucky

²¹ For example, in *Darwinula stevensoni*, up to 10⁵ individuals per m² have been observed (Van Doninck et al. 2003b) and in oribatid mites, 425 000 individuals per m² (Anderson 1978).

lineages" had already developed special mechanisms such as GPG or highly efficient DNA repair, allowing them to survive when they abandoned sex.

Interestingly, none of the three examples of ancient asexuals has developed mixed parthenogenesis, although the arbuscular mycorrhizal fungi still require further investigations, considering the evolutionary consequences of hyphal fusion and exchange of genetically different nuclei.

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